Basic Science

Protective Role for Toll-Like Receptor-9 in the Development of Atherosclerosis in Apolipoprotein E–Deficient Mice

Christine Koulis,* Yung-Chih Chen,* Christian Hausding, Ingo Ahrens, Tin Soe Kyaw, Christopher Tay, Terri Allen, Karin Jandeleit-Dahm, Matthew J. Sweet, Shizuo Akira, Alexander Bobik, Karlheinz Peter,† Alex Agrotis†

Objective—Atherosclerosis is driven by inflammatory reactions that are shared with the innate immune system. Toll-like receptor-9 (TLR9) is an intracellular pattern recognition receptor of the innate immune system that is currently under clinical investigation as a therapeutic target in inflammatory diseases. Here, we investigated whether TLR9 has a role in the development of atherosclerosis in apolipoprotein E–deficient (ApoE−/−) mice.

Approach and Results—Newly generated double-knockout ApoE−/−:TLR9−/− mice and control ApoE−/− mice were fed a high-fat diet from 8 weeks and effects on lesion size, cellular composition, inflammatory status, and plasma lipids were assessed after 8, 12, 15, and 20 weeks. All 4 time points demonstrated exacerbated atherosclerotic lesion severity in ApoE−/−:TLR9−/− mice, with a corresponding increase in lipid deposition and accumulation of macrophages, dendritic cells, and CD4+ T cells. Although ApoE−/−:TLR9−/− mice exhibited an increase in plasma very low-density lipoprotein/low-density lipoprotein cholesterol, the very low-density lipoprotein/low-density lipoprotein:high-density lipoprotein ratio was unaltered because of a parallel increase in plasma high-density lipoprotein cholesterol. As a potential mechanism accounting for plaque progression in ApoE−/−:TLR9−/− mice, CD4+ T-cell accumulation was further investigated and depletion of these cells in ApoE−/−:TLR9−/− mice significantly reduced lesion severity. As a final translational approach, administration of a TLR9 agonist (type B CpG oligodeoxynucleotide 1668) to ApoE−/− mice resulted in a reduction of lesion severity.

Conclusions—Genetic deletion of the innate immune receptor TLR9 exacerbated atherosclerosis in ApoE−/− mice fed a high-fat diet. CD4+ T cells were identified as potential mediators of this effect. A type B CpG oligodeoxynucleotide TLR9 agonist reduced lesion severity, thus identifying a novel therapeutic approach in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2014;34:516-525.)

Key Words: atherosclerosis ■ CpG ODN ■ inflammation ■ Toll-like receptor-9

Atherosclerosis was once considered a lipid storage disease. However, it is now recognized as a chronic and progressive inflammatory disease of the arterial intima. Although perturbations in cholesterol metabolism influence lesion development, progression of atherosclerosis is largely dependent on immune cells, particularly macrophages and T cells that promote inflammation by secreting proinflammatory cytokines.1

Toll-like receptors (TLRs) are a family of pattern recognition receptors that enable the innate immune system to recognize and respond to invading pathogens.2 They also detect endogenous host danger signals in the context of cancer metastasis3 and chronic inflammation,4 including atherosclerosis.2

TLR involvement in atherosclerotic lesion development was initially shown using mice lacking MyD88, an intracellular adaptor protein involved in TLR signal transduction.5 Such mice displayed reduced lesion severity in the apolipoprotein E–deficient (ApoE−/−) mouse model.6 Moreover, both TLR2 and TLR4, which are expressed on the surface of many cell types, including smooth muscle cells, endothelial cells, macrophages, and dendritic cells (DCs), have also been shown to be proatherogenic in the mouse, via mechanisms that include augmented T helper (Th1) cytokine secretion6 and, in the case of TLR4, modulation of plasma cholesterol levels.6 Along with TLR4, TLR6 has also been implicated in promoting atherogenesis via initiation of inflammation in response to oxidized low-density lipoprotein (LDL).7 Conversely, it has been shown recently that endosomally located TLR3, which senses double-stranded RNA, and TLR7, which senses single-stranded CpG ODNs, protect against atherosclerosis.8

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RNA, are antiatherogenic in the ApoE−/− mouse model. These findings indicate that specific TLRs can exert both pro- and antiatherogenic effects, and that signaling from each of these TLRs influences the development of atherosclerosis.

TLR9 is an endosomally located TLR, which senses bacterial DNA via recognition of the CpG motif. This receptor can also respond to DNA released from host cells and this recognition results in increased lipid accumulation and augmented foam cell formation. Furthermore, TLR9 is expressed by pDC within fibrofatty lesions from human atherosclerotic lesions, and ex vivo activation of TLR9 with a CpG-containing oligodeoxynucleotide results in increased IFN-α secretion and increased the cytotoxicity of CD4+ T cells toward smooth muscle cells. However, other evidence suggests that TLR9 may be protective against atherosclerosis. For example, TLR9 activation in vitro stimulates interleukin (IL)-10 production by conventional DCs, which can in turn inhibit the expression of IFN-α by pDCs. TLR9 activation has also been shown to stimulate B cells to produce IL-10, which subsequently inhibits CD4+CD25+ T-cell proliferation. Moreover, TLR9 plays a tolerogenic role in mediating the production of protective self-reactive serum IgM antibodies by peritoneal B-1b cells in lupus-prone mice to control Th17 cell development and autoimmunity. In addition, the Murphy Roths Large model of murine lupus, TLR9 signaling plays a protective role by modulating the activity of CD4+CD25+ regulatory T cells.

To define the precise role that TLR9 plays in atherogenesis, we used a newly generated double-knockout C57BL/6 mouse, in which both TLR9 and ApoE were deleted. These ApoE−/−:TLR9−/− mice were fed a high-fat diet, and effects on atherosclerotic lesion formation were compared with control ApoE−/− mice. The results suggest that TLR9 has a protective function against atherosclerosis, a finding that is supported by the reduced lesion severity observed after administration of a TLR9-stimulating oligodeoxynucleotide to ApoE−/− mice. Our findings thus suggest that TLR9 agonists may have potential for the treatment of atherosclerosis.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### TLR9 Deletion and Atherosclerotic Lesion Characteristics

DCs play key roles in the development of atherosclerosis and may affect the accumulation of CD4+ T cells. We first investigated whether DCs express TLR9 in atherosclerotic plaques. Flow cytometric analysis of enzymatically digested atherosclerotic lesions from the aortic arch from ApoE−/− mice on high-fat diet demonstrated that both conventional and pDCs express TLR9 (Figure II in the online-only Data Supplement). We therefore determined the effect of TLR9 deletion on these cells in the atherosclerotic lesions. Immunohistochemical analysis of 33D1 expression indicated that there was a significant 75% increase in the accumulation of DCs in the lesions of ApoE−/−:TLR9−/− mice (P<0.05; Figure 3A; Figure III in the online-only Data Supplement). Concomitant with the increased number of DCs, IFN-α mRNA expression was also significantly increased in atherosclerotic lesions from the ApoE−/−:TLR9−/− mice (209% increase, P<0.05; Figure 3C). Expression of IL-1β was unaffected, whereas tumor necrosis factor (TNF)-α and IL-6 tended to be increased, but the difference did not reach statistical significance (P>0.05; Figure 3C). As expected, given the increase in macrophage accumulation (Figure 1B), CD68 mRNA levels were also increased (Figure 3C).

Because DCs secrete chemokines that attract CD4+ T cells, we next determined whether the accumulation of CD4+ T cells was affected by the absence of TLR9. There was a significant 107% increase in the accumulation of CD4+ T cells in the lesions of ApoE−/−:TLR9−/− mice, as compared with the ApoE−/− mice (P<0.05; Figure 3B). Furthermore, TLR9 deletion resulted in a marked 3-fold increase in mRNA expression of TIM-3 (P<0.05; Figure 3C), a membrane protein expressed during CD4+ Th1 cell differentiation and also on CD11b+ dendritic cells.

### Results

**Effect of TLR9 Deletion on Atherosclerotic Lesion Size in ApoE−/− Mice**

To assess the effect of TLR9 on atherosclerosis, we determined lesion size in the aortic sinus of ApoE−/−:TLR9−/− mice and control ApoE−/− mice fed a high-fat diet for 12 weeks. At the end of this period, both groups of mice showed similar body weights (ApoE−/−:TLR9−/− mice, 33.4±1.6 g versus control ApoE−/− mice, 32.0±1.0 g; P>0.05). Analysis of Oil-Red O–stained sections showed that there was a significant 33% increase in lipid deposition and atherosclerotic plaque size in ApoE−/−:TLR9−/− mice, as compared with ApoE−/− mice (P<0.05; Figure 1A). In addition, macrophage accumulation, as revealed by anti-monocytes and macrophage marker-2 staining, was also significantly increased in the ApoE−/−:TLR9−/− mice (71% increase; P<0.01; Figure 1B). In contrast, smooth muscle cell content was unaffected (P>0.05; Figure 1C). The expression of 2 molecules critical for the development of atherosclerosis, vascular cell adhesion molecule-1 and monocyte chemotactic protein-1, was also unaffected by TLR9 deletion (Figure 2A and 2B), as was the size of the necrotic core (P>0.05; Figure 2C). Importantly, we also confirmed that in ApoE−/−:TLR9−/− mice exacerbated atherosclerotic lesion development occurred at an earlier time point of high-fat diet feeding (8 weeks) and persisted for 15 and 20 weeks (Figure 1A in the online-only Data Supplement).

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>DCs</td>
<td>dendritic cells</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>IL</td>
<td>interleukin</td>
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<td>pDCs</td>
<td>plasmacytoid dendritic cells</td>
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<td>TLRs</td>
<td>Toll-like receptors</td>
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<td>TNF</td>
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**Materials and Methods**

Materials and Methods are available in the online-only Supplement.
Interestingly, TLR9 deficiency was also associated with increased numbers of splenic CD4+ and CD8+ T cells (P<0.05; Figure 3D) and although there was a tendency for B2 cells to be increased (Figure 3D), this was not reflected in any difference in serum levels of IgM between the 2 mouse genotypes (data not shown). Other lymphocyte populations were largely unaffected, and monocyte/macrophage subset proportions did not differ between the 2 mouse genotypes (Figure IV in the online-only Data Supplement).

TLR9 Deletion and Serum Lipid Levels

To determine whether TLR9 influenced cholesterol homeostasis, we measured total, very LDL/LDL- and high-density lipoprotein (HDL) cholesterol as well as triglycerides in plasma. These were all elevated to a greater extent in ApoE−/−:TLR9−/− mice than in ApoE−/− mice; on average, the increases were 40%, 33%, 39%, and 76%, respectively (all P<0.05; Figure 4A–4D). However, the ratio of VLDL/LDL:HDL cholesterol was not different between the 2 mouse genotypes at any time point studied (Figure 4E; Figure IB in the online-only Data Supplement). To further investigate lipid-mediated mechanisms of atherogenesis, we conducted in vitro foam cell formation experiments. We found foam cell formation to be similar in macrophages derived from bone marrow (Figure V in the online-only Data Supplement) as well as peritoneal macrophages of ApoE−/− and ApoE−/−:TLR9−/− mice.

Contribution of CD4+ T Cells to Atherosclerosis in ApoE−/−:TLR9−/− Mice

Given that we found a greater accumulation of CD4+ T cells in atherosclerotic lesions from ApoE−/−:TLR9−/− mice, in the next series of experiments we assessed the functional contribution these cells made to atherosclerosis development. Depletion of these cells by administration of a CD4-depleting antibody during an 8-week period of high-fat diet feeding in ApoE−/−:TLR9−/− mice was confirmed by flow cytometry (Figure VI in the online-only Data Supplement; P<0.05). CD4+ T-cell depletion resulted in a reduction in lesion size to half of that observed in ApoE−/−:TLR9−/− mice receiving a non–CD4-depleting isotype control antibody for the same time period (Figure 5A; P<0.05). This reduction in lesion size was independent of any changes in serum lipid levels (Figure 5B–5F). To elucidate possible...
mechanisms by which the CD4+ T cells from ApoE−/−:TLR9−/− mice could modulate atherosclerotic lesion development, we next cultured splenic CD4+ T cells isolated from ApoE−/−:TLR9−/− mice and control ApoE−/− mice in the presence of anti-CD3 antibody for 48 hours and then assayed for secretion of specific pro- and anti-inflammatory cytokines. This analysis indicated that the CD4+ T cells isolated from ApoE−/−:TLR9−/− mice secreted elevated levels of proinflammatory TNF-α (327±11.5 pg/mL in ApoE−/−:TLR9−/− mice compared with 283±10.3 pg/mL in control ApoE−/−; mean±SEM; P<0.05; n=8) and reduced levels of anti-inflammatory IL-10 (75.5±0.9 pg/mL in ApoE−/−:TLR9−/− mice compared with 80.5±1.5 pg/mL in control ApoE−/−; mean±SEM; P<0.05; n=8) as compared with CD4+ T cells isolated from control ApoE−/− mice.

### TLR9 Agonist Reduces Atherosclerotic Lesion Development

Given the exacerbated disease phenotype of ApoE−/−:TLR9−/− mice, we next investigated whether a TLR9 agonist demonstrates any therapeutic effect in reducing atherosclerotic lesion development. Eight-week-old ApoE−/− mice fed a high-fat diet were administered either the type B CpG oligodeoxynucleotide TLR9 agonist (oligodeoxynucleotide 1668) or vehicle (PBS) for a total of 8 weeks, and then atherosclerotic lesion size was determined by Oil-Red O staining. This analysis demonstrated that in ApoE−/− mice, oligodeoxynucleotide 1668 administration significantly attenuated atherosclerotic lesion development (P<0.01; Figure 6A). Moreover, this reduced lesion development occurred in the absence of any changes in serum lipid profiles between the oligodeoxynucleotide 1668 and vehicle-treated mice (Figure 6B–6F).

### Discussion

It has been demonstrated previously that TLR9 activation can result in both proinflammatory and anti-inflammatory effects in different disease settings. TLR9 contributes to acute pancreatitis in a mouse model, and treatment with a TLR9 antagonist reduced disease severity and attenuated both neutrophil
infiltration and interleukin-1β expression. Similarly, TLR9 also promotes glomerular nephritis; deletion of TLR9 attenuated CD4+ T-cell and macrophage infiltration and reduced expression of the proinflammatory cytokines IFN-γ, TNF-α, and IL-1β, as well as the chemokine monocyte chemotactic protein-1. TLR9 has also been reported to promote liver fibrosis and acute hepatoxicity in mouse disease models. In contrast, during liver ischemia/reperfusion injury, necrotic hepatocyte-derived DNA activated conventional DCs via TLR9, thereby increasing their production of IL-10. This, in turn, reduced production of TNF-α, IL-6, and reactive oxygen species by inflammatory monocytes and attenuated ischemia/reperfusion injury. Similarly, TLR9-mediated increases in DC-derived indoleamine 2,3-dioxygenase protected against experimental autoimmune diabetes mellitus. Thus, the central role of TLR9 in many inflammatory diseases is suggestive of a possible role in atherosclerosis, but available reports would not allow foreseeing the direction of TLR9’s involvement, as either pro- or antiatherosclerotic.

In vitro data describing CpG DNA-mediated activation of macrophages stimulating foam cell formation imply that TLR9 might rather contribute to the development of atherosclerosis. However, in contrast to this, our in vivo studies demonstrate that TLR9 has a protective role against atherosclerosis in the ApoE−/− mouse model. Interestingly, similar discrepancies between in vitro proinflammatory effects and
overall in vivo atheroprotective effects have been reported for TLR3 and TLR7.9,10 These discrepancies between in vitro and in vivo data might be explained by the multitude of cell types that express TLRs and cell-specific differences in the abundance of TLR ligands and thus, in the in vivo setting individual cell type responses might be counteracted by others. Also, various diseases might differ in the overall complex response to TLR signaling. For example, postinterventional vascular remodeling is reduced by the combined blockade of TLR7 and TLR9,31 whereas genetic deficiency of TLR7, as reported by Salagianni et al,10 and TLR9, as reported here, causes acceleration of atherosclerosis. Notably, our data are consistent with recent findings indicating an emerging paradigm that endosomally located TLRs (eg, TLR3, TLR7) exert atheroprotection, whereas cell surface–located TLRs (eg, TLR2, TLR4) exert proatherosclerotic effects.6–10 The availability of clinically usable agonists and antagonists of the various TLRs has the potential to open up a new promising area of therapeutic intervention in atherosclerotic disease as exemplified in this study with a TLR9 agonist.

It is known that TLR9 stimulation of human pDCs can induce the generation of CD4+CD25+ regulatory T cells, 32 which we and others have shown to have a protective role against atherosclerosis.33–35 However, CD4+CD25+ regulatory T-cell numbers were not affected by TLR9 deletion in the present study. Nevertheless, we found increases in CD4+ and CD8+ T cells contribute to increased atherosclerotic lesion development in ApoE−/−:TLR9−/− mice independently of lipid levels. ApoE−/−:TLR9−/− mice at 8 weeks were given CD4-depleting antibody or isotype control antibody (300 μg IP, once per week) for 8 weeks while being fed a high-fat diet (n=8, each group). A, Sections stained with Oil-Red O and their quantification, demonstrating that CD4+ T-cell depletion significantly ameliorated atherosclerotic lesion development; *P<0.05 comparing ApoE−/−:TLR9−/− mice treated with anti-CD4 and isotype control antibody. Total plasma cholesterol (B), high-density lipoprotein (HDL; C), very low-density lipoprotein (VLDL)/LDL (D), triglyceride (E), and VLDL/LDL:HDL ratio (F) did not change by CD4-depleting antibody or isotype control antibody. *P<0.05 for the comparison between 2 groups. Scale bar, 200μm.

Figure 4. Toll-like receptor-9 (TLR9) deficiency and effects on plasma cholesterol and triglycerides in apolipoprotein E–deficient (ApoE−/−) mice and ApoE−/−:TLR9−/− mice fed a high-fat diet for 12 weeks. Absence of TLR9 resulted in a significant increase in total plasma cholesterol (A), reflected by increases in high-density lipoprotein (HDL; B) and very low-density lipoprotein (VLDL)/LDL (C) in the ApoE−/−:TLR9−/− mice as compared with the ApoE−/− mice. Total triglyceride levels (D) were also significantly higher in the ApoE−/−:TLR9−/− mice. The ratio of VLDL/LDL:HDL (E), however, was not significantly different between the ApoE−/−:TLR9−/− mice as compared with the ApoE−/− mice. **P<0.01; n=12; ApoE−/−:TLR9−/− mice compared with control ApoE−/− mice.

Figure 5. CD4+ T cells contribute to increased atherosclerotic lesion development in ApoE−/−:TLR9−/− mice independently of lipid levels. ApoE−/−:TLR9−/− mice at 8 weeks were given CD4-depleting antibody or isotype control antibody (300 μg IP, once per week) for 8 weeks while being fed a high-fat diet (n=8, each group). A, Sections stained with Oil-Red O and their quantification, demonstrating that CD4+ T-cell depletion significantly ameliorated atherosclerotic lesion development; *P<0.05 comparing ApoE−/−:TLR9−/− mice treated with anti-CD4 and isotype control antibody. Total plasma cholesterol (B), high-density lipoprotein (HDL; C), very low-density lipoprotein (VLDL)/LDL (D), triglyceride (E), and VLDL/LDL:HDL ratio (F) did not change by CD4-depleting antibody or isotype control antibody. *P<0.05 for the comparison between 2 groups. Scale bar, 200μm.
T cells, but not other lymphocyte populations, in the spleens of ApoE−/−:TLR9−/− mice. We have shown recently that CD8+ T cells can contribute to the development of a vulnerable atherosclerotic plaque phenotype in the ApoE−/− mouse model by perforin- and granzyme B–mediated apoptosis and promotion of necrotic core formation.36 However, in the current study, we found that necrotic core size was not different between ApoE−/− and ApoE−/−:TLR9−/− mice, suggesting that CD8+ T cells in this setting did not significantly contribute to atherosclerotic lesion characteristics. Our data point to a critical role of CD4+ T cells in atherosclerotic lesion development in the ApoE−/− mouse in the absence of TLR9. We found this lymphocyte population to be increased, not only in the spleens of ApoE−/−:TLR9−/− mice but also within the lesions of these mice. Moreover, their functional contribution to exacerbated lesion formation was shown in experiments in which the administration of a CD4-depleting antibody significantly attenuated lesion formation in ApoE−/−:TLR9−/− mice. Our additional finding that splenic CD4+ T cells from ApoE−/−:TLR9−/− mice secreted more of the proinflammatory cytokine TNF-α, and less of the anti-inflammatory cytokine IL-10, than did such splenic cells from the control ApoE−/− mice, describe a potential mechanistic link between these lymphocytes and exacerbated atherosclerosis in the ApoE−/−:TLR9−/− mice. This is of particular interest because TNF-α has long been implicated in macrophage proliferation,37 which only just recently has been identified as a major contributing factor in atherogenesis.38 This localized source of macrophages in plaques is consistent with a localized modulatory role of the innate immune system in atherosclerosis.

In our study, TLR9 deletion also resulted in upregulation of Tim-3 in atherosclerotic lesions. Tim-3 is expressed by both CD11b+ DCs and CD4+ Th1 cells, but not CD11b+ macrophages.23 Its increased expression in the atherosclerotic lesions from ApoE−/−:TLR9−/− mice is consistent with our finding of an increased abundance of these cell populations within lesions. Tim-3 ligation on DCs synergizes with TLRs in promoting inflammatory responses,23 so it is possible that enhanced Tim-3 expression contribute to the augmented atherosclerosis in the TLR9-deficient mice, possibly via the increase in IFN-α that concomitantly occurred in the lesions of the ApoE−/−:TLR9−/− mice. This possibility is consistent with a recent study showing that stimulation of TLR9 on DCs reduces IFN-α secretion.39 Several lines of evidence suggest that the elevation in IFN-α that we observed in atherosclerotic lesions of the TLR9-deficient mice (an increase in the plasma level was not seen, data not shown) can contribute to the augmentation in lesion growth. Low-dose IFN-α treatment accelerated atherosclerosis in LDL receptor–deficient mice.40 IFN-α also stimulated Ly6C+ monocyte migration to sites of inflammation and it also promoted lipid uptake by macrophages and augmented macrophage-derived foam cell formation.41 Consistent with this proatherosclerotic role of IFN-α is the finding of a recent study, which demonstrated the proatherosclerotic role of pDCs, which are known to be high producers of IFN-α.42 However, there is also a report identifying

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**Figure 6.** Toll-like receptor-9 (TLR9) activation ameliorates atherosclerotic lesion formation in apolipoprotein E–deficient (ApoE−/−) mice, independently of lipid levels. Administration of Type B CpG oligonucleotide (ODN 1668, 10 μg/kg IV, weekly) to 8-week-old ApoE−/− mice (n=8) for a period of 8 weeks resulted in significantly attenuated atherosclerotic lesion formation compared with vehicle administration (n=5), as shown by Oil-Red O–stained sections (A). Total plasma cholesterol (B), high-density lipoprotein (HDL; C), very low-density lipoprotein (VLDL)/LDL (D), triglyceride (E), and VLDL/LDL:HDL ratio (F) did not change by type B CpG oligodeoxynucleotides. **P<0.01** ApoE−/− mice administered with ODN 1668 compared with ApoE−/− mice without ODN 1668 (vehicle-only) for 8 weeks. Scale bar, 200 μm.

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pDCs and IFN-α as being atheroprotective, indicating further studies on the emerging but potentially complex role of DCs in atherosclerosis are needed.

At present, we cannot clearly define which cell type is primarily responding to TLR9 in atherosclerotic lesions, although several lines of evidence suggest that it may be pDCs. Our study indicates that pDCs in atherosclerotic lesions express TLR9. Furthermore, recent studies indicate that high concentrations of CpG oligodeoxynucleotides stimulate a tolerogenic TLR9-TIR domain containing adapter inducing interferon-β (TRIF) pathway in pDCs; such DCs, for example, prevent airways inflammation. It is possible that such a pathway is also stimulated in atherosclerotic lesions. Unfortunately, there is no specific surface marker that would allow the identification of tolerogenic DCs in atherosclerotic plaques. As to the source of TLR9 stimulatory oligonucleotides, recent studies indicate that DNA complexes on the surface of apoptotic cells are capable of activating TLR9; apoptotic cells are present in significant numbers in lesions. Although TLR9 activation also stimulates naïve B cells to proliferate and differentiate into IgM-secreting cells and synthetic CpG oligodeoxynucleotides are known to augment B-cell activating factor mediated IgG secretion, plasma IgM and IgG levels were unaffected by TLR9 deletion, making it unlikely that an effect on B cells contributes to the atheroprotective effects of TLR9.

Our data raise the intriguing possibility that synthetic TLR9 agonists may represent a novel therapeutic approach in atherosclerosis. Previous studies have shown that the therapeutic potential of CpG oligodeoxynucleotides is likely to be highly dependent on CpG oligodeoxynucleotides type. Interestingly, the same type B CpG oligodeoxynucleotide as used in our study has been shown to ameliorate intestinal inflammation and to decrease the severity of colitis in a mouse model of inflammatory bowel disease. Moreover, type B CpG oligodeoxynucleotides administered to mice before myocardial ischemia followed by reperfusion was able to significantly decrease infarct size and improve cardiac function. Overall, CpG oligodeoxynucleotides are promising drug candidates with many human trials, including phase 3, currently ongoing.

It has been shown previously that deletion of TLR4 attenuated the development of atherosclerosis via decreases in IL-12, monocyte chemoattractant protein-1, macrophage numbers, and cyclo-oxygenase-2. These effects were discussed as being independent of cholesterol levels. More recently, however, it has been proposed that TLR4 exerts at least part of its proatherosclerotic capacity via an increase in plasma cholesterol levels, specifically in cholesterol-rich VLDL/LDL particles. Interestingly, this increase in VLDL/LDL cholesterol plasma levels was not associated with changes of HDL cholesterol. Therefore, the proatherosclerotic effects of TLR4 are likely to be associated with the detected proatherogenic increase of VLDL/LDL cholesterol. In contrast, our study shows that the increase in plasma levels of VLDL/LDL cholesterol is paralleled by an increase in HDL cholesterol, which has been shown to be atheroprotective in mice and humans. Thereby the VLDL/LDL/HDL cholesterol ratio, which is seen as strong determinant of cardiovascular risk, remains unchanged. Furthermore, the importance of direct, cholesterol-independent inflammatory mechanisms is further underscored by our demonstration that both CD4+ T-cell depletion and type B CpG oligodeoxynucleotide administration were able to attenuate TLR9-associated lesion development in the absence of any alterations in VLDL/LDL cholesterol plasma levels. In summary, the changes observed in the lipid profile in ApoE−/−:TLR9−/− mice point to an interesting role of TLR9 in cholesterol metabolism, which warrants further studies. Although our findings suggest a tolerogenic mechanism dependent on TLR9 activation of pDCs as being important in mediating the atheroprotective effects of TLR9, the fact that TLR9 also regulates plasma lipid levels suggests that a lipid-dependent mechanism may also contribute. Because DCs are also known to modulate hypercholesterolemia, it is tempting to speculate that TLR9 may also initiate a yet to be identified effect in DCs that regulates hypercholesterolemia.

In summary, this study advances our knowledge of the important but complex role of TLRs in atherosclerosis. Using a genetic and a pharmacological approach, we can demonstrate a protective role of TLR9 in atherosclerosis and we describe the modulation of cell numbers and inflammatory phenotype of CD4+ T cells as potential mechanisms. Moreover, we have identified a novel and attractive therapeutic approach to attenuate progression of atherosclerotic disease based on the use of specific TLR9-activating CpG oligodeoxynucleotides, which belong to a promising drug group that is already in clinical development.

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Disclosures
None.

References


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Significance

Atherosclerosis is the underlying pathology of diseases, such as coronary artery disease, resulting in major overall mortality and morbidity. It is a chronic inflammatory disease of the arterial wall, in which the innate immune system has a pivotal contribution. Toll-like receptor-9 (TLR9) is a major intracellular innate immune receptor that has attracted major interest and is currently clinically tested as a therapeutic target of inflammatory diseases. Using apolipoprotein E-deficient mice fed with high-fat diet during periods of 8 to 20 weeks as a model of atherosclerosis, we used a genetic (TLR9 deletion) and a pharmacological approach (TLR9 agonist) to demonstrate a protective role of TLR9 in atherosclerosis. We describe the modulation of cell numbers and inflammatory phenotype of CD4+ T cells as a potential mechanism. Moreover, we have identified a novel and attractive therapeutic approach to attenuate progression of atherosclerotic disease based on the use of TLR9-activating CpG oligodeoxynucleotides.
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A protective role for TLR9 in the development of atherosclerosis in ApoE-deficient mice

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Running title: TLR9 protects from atherosclerosis

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Supplemental Figure I. Genetic TLR9 deletion is associated with increased atherosclerotic lesion development at both early (8-week) and late (15- and 20-week) time points. Data represent aortic lesion size assessed from the mean area of Oil-Red O staining of aortic sinus cross-sections from high fat diet-fed control ApoE−/− mice (white bars) and ApoE−/−:TLR9−/− mice (black bars) (A). At each time point, VLDL/LDL:HDL ratios were not different between the two mouse strains (B). *P<0.05; **P<0.01 ApoE−/−:TLR9−/− mice compared with control ApoE−/− mice (N≥8, each group).
Supplemental Figure II. Flow cytometric analysis of TLR9 expression of dendritic cells within the atherosclerotic lesions of ApoE−/− mice. Aortic arches from ApoE−/− mice were harvested and enzymatically digested to single cell suspensions and permeabilized. Cells were stained for CD11c and intracellular TLR9 or isotype control. TLR9 was expressed in both conventional and plasmacytoid DCs.
Supplemental Figure III. IgG 2b staining (Isotype control for 33D1 staining) of the atherosclerotic lesion from ApoE\(^{-/-}\) and ApoE\(^{-/-}:\text{TLR9}^{-/-}\). Scale bar indicates 50µm.
Supplemental Figure IV. ApoE<sup>−/−</sup>:TLR9<sup>−/−</sup> mice exhibit similar levels of ‘inflammatory’ monocytes/macrophages compared to ApoE<sup>−/−</sup> mice fed a high fat diet. CD11b<sup>+</sup> and CD115<sup>+</sup> double positive cells were first gated (data not shown) then sub-gated as Ly6C<sup>hi</sup>, Ly6C<sup>int</sup> and Ly6C<sup>lo</sup> monocyte subsets from spleens of 12 weeks old ApoE<sup>−/−</sup>:TLR9<sup>−/−</sup> and ApoE<sup>−/−</sup>:TLR9<sup>+/+</sup> mice. Results are expressed as mean percentage of CD115<sup>+</sup>CD11b<sup>+</sup> monocyte subtypes ± SEM from 8 mice per group.
Supplemental Figure V. Quantification of PBS, LDL and PMA+LDL treated bone marrow-derived macrophages from ApoE−/− and ApoE−/−:TLR9−/− mice. Macrophages were incubated for 2 days with LDL (500µg/ml) with or without PMA (50ng/ml). Following incubations, macrophages were rinsed, fixed and stained with Oil-red O for quantification of lipid content.
Supplemental Figure VI. Reduced CD4$^+$ T cell accumulation in spleens of ApoE$^{-/-}$ :TLR9$^{-/-}$ mice after administering a CD4-depleting antibody. ApoE$^{-/-}$ :TLR9$^{-/-}$ mice at 8 weeks of age were administered either CD4-depleting antibodies or isotype control antibodies (300µg, I.P., once per week) for 8 weeks whilst being fed a high-fat diet. Bar graphs represent the proportion of splenic lymphocyte subsets assessed by flow cytometric analysis at the end of the treatment regimen, demonstrating the significant reduction in CD4$^+$ T cells in the CD4-depleting antibody-treated group. *$P<0.05$ comparing CD4-depleting with isotype control antibody-treated ApoE$^{-/-}$ :TLR9$^{-/-}$ mice (N=8, each group).
Materials and Methods

A protective role for TLR9 in the development of atherosclerosis in ApoE-deficient mice

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**Generation of ApoE⁻/--:TLR9⁻/-- “double-knockout” mice**

TLR9⁻/-- mice on a C57Bl/6 background were crossed with ApoE⁻/-- mice on a C57Bl/6 background, and F2 mice were genotyped to obtain ApoE⁻/--:TLR9⁻/-- “double-knockout” (DKO) mice homozygous for both the TLR9 mutant allele and the ApoE mutant allele. To confirm the double-knockout, tail tip DNA (Qiagen columns) was isolated and subjected to PCR using Invitrogen PCRx enhancer methodology with previously published primer pairs. For TLR9 genotyping, ¹ a common sense primer (GCAATGGAAAGGACTGTCCACTTTGTG) in the 2nd intron was used in combination with an antisense primer either (i) specific for the downstream wild type exonic sequence (GAAGGTTCTGGGCTCAATGGTCATGTG) or (ii) specific for the downstream targeting construct sequence (ATCGCCTTCTATCGCCTTCTTGACGAG). The fragments amplified by these primers were a 1108 bp wild-type TLR9 fragment, and a 1158 bp mutant TLR9 fragment. For ApoE genotyping, primers used were those specified by Jackson Laboratories, in which a common sense primer (olMR0180, AGCCTTAAACTTACTCTACACA) was used in combination with an antisense primer either (i) specific for the downstream wild-type exonic sequence (olMR0181, TGTGACTTGGGAGCTCTGCAGC) or (ii) specific for the downstream targeting construct sequence (olMR0181, GCCGCCCCGACTGCATCT). The fragments amplified by these primers were a 155 bp wild-type ApoE fragment and a 245 bp mutant ApoE fragment. Controls for PCR included known wild-type and mutant tail DNA for TLR9 and ApoE, and water as a negative control.

**Animal experiments**

DKO ApoE⁻/--:TLR9⁻/-- male mice and control ApoE⁻/-- male mice were fed a high-fat diet containing 21% butter fat and 0.15% cholesterol (Specialty Feeds) from 8 weeks of age for a period of 8 to 20 weeks. The mice were then euthanased with an overdose of pentobarbitone (120mg/kg i.p.), blood was collected by cardiac puncture and aortic sinus and arch collected for histology and molecular studies, respectively. All experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee.

**Quantification of atherosclerotic lesions**

The aortic sinus was dissected from mice, embedded in OCT compound (Tissue-tek) and frozen at -80°C. Six-μm sections of the aortic sinus were analyzed for lesion size, defined as the cross-section surface area of Oil-Red O staining within the aortic intima. Mean lesion size for each mouse was calculated from measurement of cross sections from every 60μm of the first 180μm in the ascending aorta, starting from the aortic cusp.²

**Immunohistochemistry**

Sections of the aortic sinus were used for immunohistochemistry as previously described³ to assess macrophage accumulation (MOMA-2), CD4⁺ T-lymphocytes, dendritic cells (33D1)⁴, vascular smooth muscle cells (α-smooth muscle actin), necrotic core area (via H&E), VCAM-1 and MCP-1 expression. Briefly, sections were fixed in cold (-20°C) acetone for 20 min. They were then incubated in 3% hydrogen peroxide in PBS, 10% normal serum and biotin/avidin blocking reagents (Vector Laboratories) for 30, 20, and 15 minutes, respectively. Sections were then incubated for 1 hr with primary antibodies in serum: rat anti-mouse MOMA-2 (1:100; cat...
#MCA519G; Serotec), rat anti-mouse VCAM-1 (1:50; cat # 550547; BD Pharmingen), rabbit anti-rat MCP-1 (1:100; cat #ab7202; abcam), rat anti-mouse 33D1 (1:50; cat #14-5884-82; ebioscience), rat anti-mouse CD4 (1:20; cat #550280; BD Pharmingen) or rabbit anti-human α-smooth muscle actin (1:100; cat # ab 5694; abcam). Following the incubation, sections were washed and incubated with the appropriate secondary antibody (biotinylated mouse anti-rat (1:200; BD Pharmingen: cat #550325), biotinylated mouse anti-Armenian hamster (1:200; ebioscience; cat #13-4113-85) or biotinylated anti-rabbit (1:200; Vector Labs; cat #BA-1000)) for 40 minutes, after which samples were then incubated with streptavidin horseradish peroxidise complex (Vector Laboratories). Antigens were visualised using 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. For each mouse, quantification is given as the average of three or more sections of aortic sinus taken at 60µm intervals. For quantification of immunopositive cells (CD4 and MCP-1), the numbers of stained cells in cross sections were determined by counting under the microscope; atheroma size was also determined and then immunopositive cells were expressed as number of cells per mm² of total atheroma area. The quantification of smooth muscle cells (α-smooth muscle actin), dendritic cells (33D1) and macrophages (MOMA-2) in lesions was performed by measuring the immunostained areas and the result was expressed as a percentage relative to the total atheroma area to take into account different lesion sizes.

Analysis of gene expression by RT-PCR
Total RNA was extracted from tissues, resuspended in sterile water and DNase treated (1U, Promega) for 15 minutes at 37°C to remove any contaminating DNA, as described previously. Subsequently, 0.1 volume of 3M sodium acetate and an equal volume of isopropanol were added, and the RNA was sedimented by centrifugation. The RNA pellet was washed by resuspension in 70% aqueous ethanol followed by centrifugation, and then air-dried for 30 minutes. It was then dissolved in sterile water and quantitated by spectrophotometry at 260nm after which it was reverse transcribed using TaqMan methodology (Applied Biosystems). Then 20ng of cDNA was used for real time PCR to determine the expression of specific genes using SYBR Green PCR mix (Applied Biosystems) and the ABI Prism version 2.04 using the 7500 PCR instrument. Each amplification reaction was performed in duplicate using 18S as the internal housekeeping control gene. Relative amounts of mRNA for specific genes in lesions from control ApoE+/+ :TLR9+/+ mice and DKO ApoE-/- :TLR9-/- mice were calculated using comparative C_{T} values. The sequences of the primers used for CD68, TIM-3, IL-1β, TNF-α and IL-6 amplification were as previously described. For IFN-α amplification, the primer sequences were as follows: sense, 5'-TCCTCAGACTCATAACCTCAGGAA-3'; antisense, 5'-GGGAGAGTCTCCTCATTTGTACCA-3'.

Flow cytometry
Lymphocytes were analyzed using flow cytometry as previously described. Fluorochrome-labeled antibodies (all from BD Biosciences unless otherwise stated) used were anti-CD22 (PE-Cat# 553384), anti-CD5 (APC-Cat# 550035), anti-CD25 (APC-Cy7-Cat# 557658), anti-CD4 (Pacific Blue-Cat# MCD0428, Caltag Laboratories), anti-CD8 (PerCP-Cat# 553036), anti-TCR-β (FITC-Cat# 11-596185, ebioscience), anti-NK1.1 (PE-Cy7-Cat# 552878) and anti-foxp3 (PE-Cat# 12-5773-82, ebioscience). For surface markers, single cell suspensions were stained with multiple antibodies at 4°C for 30 minutes, then washed and resuspended in PBS with
1% FCS. For regulatory T cells, anti-CD4 and anti-CD25 stained cells were fixed, permeabilized and further stained with an anti-foxp3 antibody. FACS Canto II (BD Biosciences) was used to collect data from different fluorochrome-labeled cells. FACS Diva software (BD Biosciences) was used to analyze the data. For Ly6C analysis of monocytes, fluorochrome-labeled antibodies were used as following: anti-CD115 (APC-Cat#17115280), anti-CD11b (PE-Cat#12011281) and Ly6C (V450-BD-Cat#560594).

Enzymatic digestion of tissues for flow cytometry
ApoE−/− mice fed with high fat diet for 10 weeks were sacrificed and perfused with PBS. Aortic arches were freed of all visible adventitial fat under a 6× dissecting microscope and then digested in 2.5 ml -5 ml of enzyme cocktail, containing 450 U/ml collagenase type I, 250 U/ml collagenase type XI, 120 U/ml hyaluronidase type I-s, 120 U/ml DNAse 1 in 1x HBSS(with Ca and Mg). Flow cytometry analysis was performed on FACS Canto II (BD Biosciences), data analyzed using FlowJo software (Tree Star Inc., Ashland). For dendritic cells analysis, all events were acquired, 500,000 events were read. Gating was performed for live, CD11c+ DC events then TLR9 (FITC-Cat#11-9093-80) or Isotype-FITC.

In-vitro foam cell formation assay
Thioglycollate-elicited peritoneal macrophages were isolated 96 hours after intraperitoneal thioglycollate injection from ApoE−/− and ApoE−/−:TLR9−/− mice. Bone marrow cells were harvested from femurs and tibias of ApoE−/− and ApoE:TLR9 deficient mice. Isolated bone marrow cells were cultured in the presence of L-cell conditioned-media to differentiate cells into macrophages. Macrophages were then cultured onto 8-well chamber slides and allowed to adhere for 24 hours. Thioglycollate-elicited peritoneal macrophages as well as bone marrow derived macrophages were then treated for 48 hours with either PBS, LDL (500µg/ml), or PMA + LDL (50ng/ml, 500µg/ml respectively). Cells were then washed, fixed and stained with Oil red O, according to manufacturer's instructions (Lifeline). Images were quantified using an FSX microscope (Olympus) and staining quantified using ImagePro 6.0 software.

Plasma cholesterol and triglycerides
Plasma VLDL/LDL- and HDL-cholesterol were determined enzymatically using a Cobas Mira Plus Autoanalyzer and an HDL and VLDL/LDL cholesterol quantification kit (BioVision, Mountain View, CA). Plasma triglycerides were determined using a triglyceride quantification kit (BioVision).

CD4 T cell depletion
DKO ApoE−/−:TLR9−/− male mice at 8 weeks of age were assigned randomly to two groups. Mice were given CD4 depleting antibodies or isotype control antibodies (300µg, I.P., once per week) for 8 weeks. During this period, mice were fed the high-fat diet described above. At the age of 16 weeks, mice were culled and the aortic sinus was collected for lesion analysis, blood was collected for plasma cholesterol and triglyceride analysis, and inguinal and para-aortic lymph nodes and spleen were collected for analysis of cell populations by flow cytometry.
**CD4 T cell cytokine secretion**

Splenic CD4+ T cells from DKO ApoE⁻⁻:TLR9⁻⁻ or ApoE⁻⁻ mice were isolated by a commercial kit (MACS, CD4⁺ T cell isolation kit II). Cells were then incubated at a concentration of 10⁶ cells/ml cell culture medium (RPMI-1640, 10% fetal calf serum). Cells were stimulated for 48 hours in anti-CD3 (2.5µg/well) coated wells. Supernatants were collected and secreted levels of TNF-α and IL-10 were measured by ELISA (eBioscience).

**IgM ELISA**

Plasma immunoglobulin IgM were measured by enzyme linked immunosorbent assay (ELISA). Nunc Maxisorp 96-well ELISA plates were coated with 50µl of 2mg/ml goat anti-mouse Ig antibody (Cat# 1010-01, Southern Biotech) overnight at 4°C. After addition of 50µl of plasma diluted at 10⁵, the plates were incubated for 1 hour at room temperature. Secondary antibody incubation was done for 1 hour at room temperature with HRP-conjugated goat anti-mouse IgM (Cat GM-90P, ICL). TMB substrate was used for color development. After stopping the reaction, optical density was measured at 450nm wavelength using a conventional ELISA reader.

**CpG ODN administration**

The type B CpG oligodeoxynucleotide (ODN-1668) was administered at a dose of 10µg/kg, I.V., once per week to ApoE⁻⁻ male mice at 8-weeks of age for a period of 8 weeks. Another group of ApoE⁻⁻ male mice received vehicle administration for the same time period. During this period, mice were fed the high-fat diet described above. At the age of 16 weeks, mice were culled and aortic sinuses and blood was collected for lesion analysis, and plasma cholesterol and triglyceride analysis, respectively.

**Statistical analysis**

Statistical analyses were performed using Student’s t-test, when data followed a normal distribution, or Mann-Whitney U test when data did not follow a normal distribution, using the software GraphPad Prism v4.01. Differences were considered statistically significant when P<0.05.

**References for Materials and Methods**


