Hyperlipidemia, inflammation and atherosclerosis : roles of apolipoprotein C1 and cholesteryl ester transfer protein

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CHAPTER 5

Apolipoprotein CI is Crucially Involved in Lipopolysaccharide-Induced Atherosclerosis Development in ApoE-Knockout Mice

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Submitted

Abstract

Lipopolysaccharide (LPS) that is released from Gram-negative bacteria upon multiplication or lysis aggravates atherosclerosis in humans and rodents by induction of chronic inflammation via Toll-like receptors. Since apolipoprotein CI (apoCI) enhances the LPS-induced inflammatory response in macrophages *in vitro* and in mice, we investigated the effect of endogenous apoCI expression on LPS-induced atherosclerosis in mice.

Hereto, 12 week old *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice were weekly injected intraperitoneally with LPS (50 μg) or vehicle for a period of 10 weeks, and atherosclerosis development was assessed in the aortic root. LPS administration did not affect atherosclerotic lesion area in *apoe*$^{-/-}$*apoc1*$^{-/-}$ mice, but increased it in *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. In fact, apoCI expression increased the LPS-induced atherosclerotic lesion area by 60% (P<0.05), concomitant with an enhancement of the LPS-induced plasma levels of fibrinogen and E-selectin, indicating a higher inflammatory state, both systemically and at the level of the vessel wall.

We conclude that apoCI is crucially involved in the LPS-induced atherosclerosis in *apoe*$^{-/-}$ mice, which mainly relates to an increased inflammatory state. We anticipate that apoCI plasma levels contribute to accelerated atherosclerosis development in individuals that suffer from chronic infection.

Introduction

Cardiovascular disease (CVD) is the principal cause of death in Europe, the United States and much of Asia.The main cause of CVD is atherosclerosis.[1-3] Though hypertension and hyperlipidemia are common risk factors for atherosclerosis, it is also evident that bacterial infections worsen the outcome of atherosclerosis by maintaining a heightened state of inflammatory response, thus propagating this inflammatory disease.[1-4] Lipopolysaccharide (LPS) is a highly inflammatory constituent of the outer membrane of Gram-negative bacteria. When these bacteria multiply or lyse, LPS is released and then activates the MD-2/Toll-like receptor 4 (TLR4) receptor complex on endothelial cells, neutrophils and macrophages, resulting in a proinflammatory response through the activation of the nuclear factor (NF)κB-pathway.[5]

TLR4 is expressed in macrophages and endothelial cells within atherosclerotic lesions,[6;7] and indeed, impaired LPS signaling in human carriers of the TLR4 polymorphism Asp299Gly or Thr399Ile is associated with LPS hyporesponsiveness[8] and the Asp299Gly mutation is associated with decreased CVD.[4] Conversely, infection with the Gram-negative bacterium *Chlamydia pneumoniae* has been associated with increased CVD in humans.[9-11]

In rodents, bacterial infection and LPS also promote atherogenesis. For example, local delivery of *C. pneumoniae* in the vessel wall of the carotid arteries increases the development of atherosclerosis in low-density-lipoprotein receptor gene deficient (*ldlr*$^{-/-}$) mice,[12] and infection with *C. pneumoniae* accelerates the development of atherosclerosis in *apolipoprotein e* gene deficient (*apoe*$^{-/-}$), *ldlr*$^{-/-}$, and *APOE*3-Leiden* transgenic mice.[13-15] In addition, LPS accelerates intimal lesion development in a peri-adventitial cuff model in wild-type mice, which is largely decreased when TLR4 expression is absent.[16] Furthermore, repeated intravenous (iv) and intraperitoneal (ip) administration of LPS accelerate atherosclerosis in rabbits and *apoe*$^{-/-}$ mice, respectively.[17;18]

Until recently, apolipoprotein CI (apoCI) was mainly known for its role in lipoprotein metabolism. ApoCI circulates in plasma with a concentration of 6 mg/dl and is mainly bound to the lipoproteins very-low-density-lipoproteins (VLDL), chylomicrons, and high-density-lipoproteins (HDL).[19] ApoCI inhibits lipoprotein lipase (LPL),[20;21] and endogenous apoCI expression was associated with modest hyperlipidemia in *apoe*$^{-/-}$ mice.[21] Recently, we have discovered that apoCI strongly binds to LPS, thereby augmenting the inflammatory response to LPS and the Gram-negative bacterium *Klebsiella pneumoniae* in mice *in vivo* and to LPS in macrophages *in vitro*.[22]

In the present study, we investigated the effect of endogenous apoCI on the development of LPS-induced atherosclerosis by using *apoe*$^{-/-}$*apoc1*$^{-/-}$ versus *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. We found that the LPS-induced atherosclerosis is enhanced in the apoCI expressing mice, associated with a higher inflammatory state achieved with LPS.

Methods

Animals

Apoe$^{-/-}$*apoc1*$^{-/-}$[23] and *apoe*$^{-/-}$[24] mice have been generated as described previously, and back-crossed at least eight times to the C57Bl/6 background. *Apoe*$^{-/-}$*apoc1*$^{-/-}$ mice were crossed with *apoe*$^{-/-}$ mice to ultimately generate *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ littermates. Female mice were used for experiments, housed under standard conditions with a 12 h light cycle (7.00 am – 7.00 pm), and fed *ad libitum* with regular chow. Animal experiments were performed at the Gaubius Laboratories of The Netherlands Organization for Applied Scientific Research-Quality of Life and all experimental protocols were approved by the local Ethics Committee for Animal Experiments.

Analysis of LPS response in time

At the age of 12 weeks, *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ littermates (n=8 per genotype) received an i.p. injection of LPS (50 µg; *E. coli* serotype 055:B5, Sigma, St Louis, MO) in 200 µl of PBS. Twenty-four hours prior the LPS injection (t=0) and every 24 h thereafter for a period of a week, 35 µl of blood was collected after a 4 h fast for the measurement of total cholesterol (TC), fibrinogen, and E-selectin. At t=0, 24 h after the LPS injection, and a week after the injection, triglyceride (TG) levels were also measured.

Atherosclerosis study

At the age of 12 weeks, *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ littermates received weekly i.p. injections of LPS (50 µg) in 200 µl of PBS, or PBS alone (vehicle) (n=8-11 per genotype per group), exactly as described for *apoe*$^{-/-}$ mice before.[18;25] Plasma lipid analysis of these mice was carried out 1 week before the start of the injections and 24 h after the first, fifth, and tenth injection. Twenty-four hours after the first injection, lipid distribution over lipoproteins was assessed. In plasma obtained 24 h after the first, fifth, and tenth injection, inflammatory parameters were measured.

Plasma lipid and lipoprotein analysis

Blood was collected by tail bleeding into capillary tubes. The tubes were placed on ice, centrifuged at 4°C, and the obtained plasma was snap-frozen in liquid nitrogen. Plasma for lipid and lipoprotein analysis was stored at –20°C and plasma for analysis of inflammatory parameters was stored at -80°C. Plasma was assayed for TC and TG using commercially available enzymatic kits 236691 and 1488872 (Roche Molecular Biochemicals, Indianapolis, IN), respectively. For determination of the lipid distribution over plasma lipoproteins by fast performance liquid chromatography (FPLC), 50 µl of pooled plasma (8-11 mice per pool) was injected onto a Superose 6 HR 10/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µl/min PBS, 1 mM EDTA (Sigma), pH 7.4. Fractions of 50 µl were collected and assayed for TC and TG as described above.

Plasma analysis of fibrinogen and E-selectin

Plasma fibrinogen levels were determined using a homemade ELISA, exactly as described previously,[26] and plasma E-selectin levels using commercially available ELISA (R&D Systems, Europe).

Atherosclerotic lesion analysis

For determination of atherosclerosis development, mice were sacrificed at 24 h after the tenth injection. Hearts were isolated and fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin, and were cross-sectioned (5 µm) throughout the entire aortic root area. Per mouse, 4 sections with 40 µm intervals were used for quantification of atherosclerotic lesion area. Sections were routinely stained with hematoxylin-phloxine-saffron (HPS). Lesion area was determined using Image-Pro Plus version 3.0 analysis software (Media Cybernetics, USA). Subsequently, sections were stained with the rabbit anti-mouse macrophage antibody (AIA-31240, dilution 1:1.000; Accurate Chemical and Scientific, Westbury, NY), the monoclonal mouse anti-smooth muscle cell (SMC) α-actin antibody (clone 1A4, dilution 1:800; Sigma), or the rat anti-human CD3 antibody (Serotec, dilution 1:1.000) as a first antibody. Biotinylated donkey anti-rabbit antibody (dilution

1:3.000; Vector Laboratories, Burlingame, CA), horse anti-mouse antibody (dilution 1:400; Vector Laboratories), or goat anti-rat antibody were used as secondary antibodies, respectively, followed by incubation with horseradish peroxidase–labeled avidin-biotin complex. Peroxidase activity was visualized with Nova Red (Vector Laboratories). Collagen was stained using Sirius red (Chroma-Gesellschaft, Stuttgart, Germany). Macrophage (AIA-31240–positive), SMC (α-actin-positive), and collagen (Sirius red-positive) lesion areas were quantified using Image-Pro Plus version 3.0 analysis software (Media Cybernetics, USA) and expressed as total area. CD3+ T-cells were counted per cross-section and expressed as total number of T-cells per individual lesion. After counterstaining of the AIA-31240 (macrophage) stained sections with hematoxylin, adherent monocytes were counted and expressed as number of monocytes per cross-section.

Statistical analysis

The Mann-Whitney nonparametric test for two independent samples was used to define differences between data sets from experimental groups. The criterion for significance was set at $P<0.05$. Statistical analyses were performed using SPSS 11.5 (SPSS Inc, Chicago, USA).

Results

Effect of apoCI on plasma lipid levels upon LPS injection

To examine the effect of LPS on plasma lipid levels, we measured the plasma levels of TC and TG before and during a period of seven days after a single LPS injection (Figure 1). LPS injection increased TC levels in both *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice at 24 h after the LPS injection by ~2.5 mmol/l. TC levels decreased thereafter to reach its starting value after 7 days (Figure 1). LPS only modestly increased the levels of TG by ~0.1 mmol/l, which returned also to the starting values within 7 days (results not shown). The expression of apoCI apparently did not affect the total LPS-induced increase in TC (Figure 1) and TG levels (results not shown).

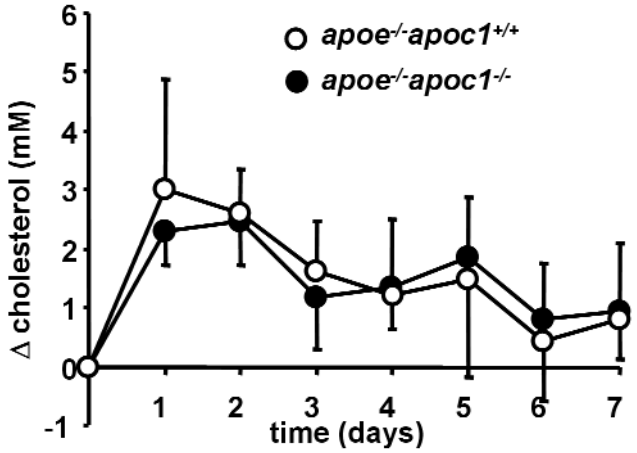


Fig. 1. Effect of apoCI on plasma cholesterol levels after lipopolysaccharide (LPS) injection. Twelve week old *apoe*$^{-/-}$*apoc1*$^{-/-}$ (black circles) and *apoe*$^{-/-}$*apoc1*$^{+/+}$ (white circles) mice received an injection of LPS (50 µg). Twenty-four hours prior to the LPS injection (t=0) and every 24 h thereafter for a period of 7 days, plasma samples were taken after a 4 h-fast. Total cholesterol (TC) was measured and the represented values indicate the increase, compared to t=0. The basal levels of TC were 7.0±0.5 and 9.3±1.8 mmol/l in *apoe*$^{-/-}$*apoc1*$^{-/-}$ mice and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice, respectively. Values are represented as means ± SD. n=8.

We next investigated the effect of repeated injections of LPS or vehicle on plasma TC and TG levels in *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. LPS or vehicle were injected weekly for a period of ten weeks, and plasma lipid levels were determined 24 h before the first injection and 24 h after the first, fifth, and tenth injection. The lipid response to the first injection was similar compared to that observed in the previous experiment. The response declined somewhat after the following injections up to ~50% after the tenth injection, indicating that repeated LPS injections led to tolerance to some extent (not shown). Over the whole period of these injections, LPS increased the TC exposure for both genotypes by only ~5%, indicating that the transient increases in TC levels as a consequence of LPS injections only contributed marginally to the total TC exposure. We also examined the effect of LPS and vehicle on cholesterol distribution over various lipoproteins in this experiment at 24 h after the first injection. It appeared that the LPS-induced increased levels of TC in mice of both genotypes were mainly confined to IDL/LDL and HDL (Figure 2A and 2B). TG levels in the various lipoprotein fractions were only marginally affected (results not shown).

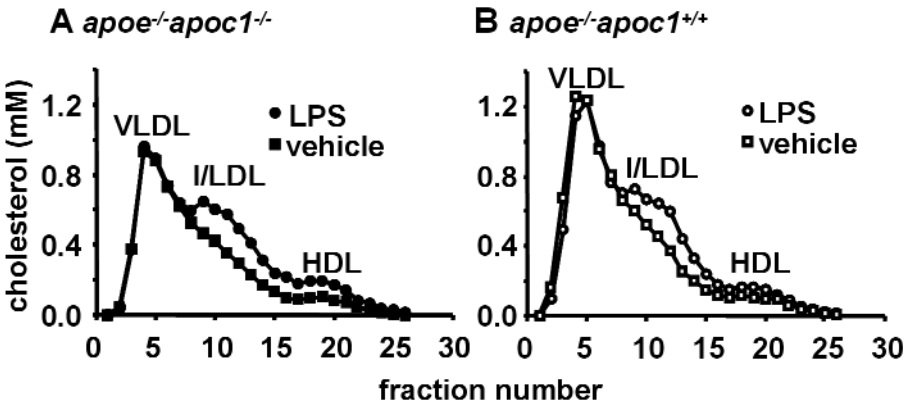


Fig. 2. Effect of apoCI on cholesterol distribution over lipoproteins after LPS. Twelve week old *apoe*$^{-/-}$*apoc1*$^{-/-}$ (A) and *apoe*$^{-/-}$*apoc1*$^{+/+}$ (B) mice received an injection of LPS (50 µg) (circles) or vehicle (squares). Twenty-four hours after the injection, plasmas from 4 h-fasted mice, injected with either vehicle or with LPS were pooled (n=8-11 per pool). Lipoproteins were size-fractionated by fast performance liquid chromatography on a Superose-6 column, and the individual fractions were assayed for cholesterol.

Effect of apoCI on plasma inflammatory markers upon LPS injection

Concomitantly with the investigation of the effect of apoCI on the lipid response induced by a single LPS injection in time as described above, we investigated the extent and duration of the LPS-induced inflammation, and the effect of apoCI on it. Hereto, we measured plasma levels of fibrinogen and E-selectin, before and during a period of 4 days after a single LPS injection into *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. The basal plasma levels of fibrinogen and E-selectin before LPS injection (t=0) were not different between *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice (Table 1). LPS injection resulted in a marked increase in fibrinogen and E-selectin in both *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. Twenty-four hours after injection, plasma levels of these inflammation markers were maximal. Both markers decreased thereafter gradually and reached their starting value after a period of ~4 days (Figure 3A and 3B). ApoCI expression significantly augmented the LPS-dependent inflammatory response with respect to both plasma markers (significant at 2 days after injection; Figure 3A and 3B).

Fibrinogen and E-selectin were also measured after repeated injections of LPS and vehicle. The response to the first injection was similar as described

Mouse genotype	Fibrinogen (mg/ml)	E-selectin (ng/ml)
apoe$^{-/-}$*apoc1*$^{-/-}$	1.23 ±0.28	96.2±5.9
apoe$^{-/-}$*apoc1*$^{+/+}$	1.26 ±0.10	91.7±13.7

Table 1. Effect of endogenous apoCI expression on basal plasma levels of inflammatory markers in *apoe*$^{-/-}$ mice. Blood was obtained from 4 h fasted *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. Plasma levels of fibrinogen and E-selectin were measured using ELISA. Values are represented as means ± SD. n=8.

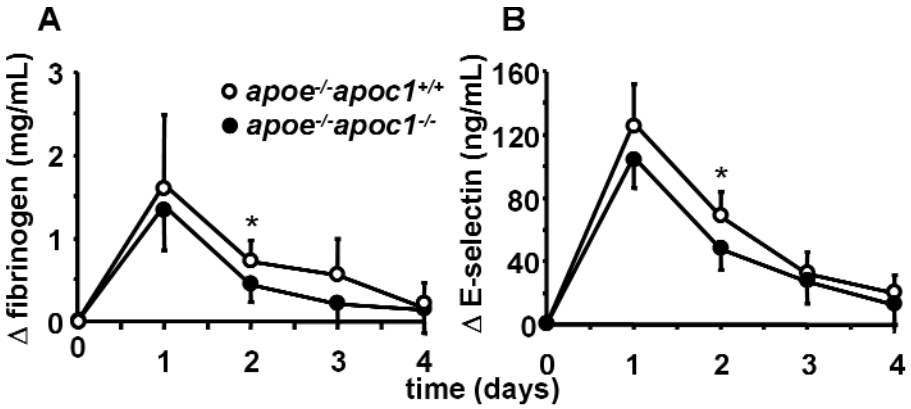


Fig. 3. Effect of apoCI on plasma inflammatory markers after LPS injection. Twelve week old *apoe*$^{-/-}$*apoc1*$^{-/-}$ (black circles) and *apoe*$^{-/-}$*apoc1*$^{+/+}$ (white circles) mice received an injection of LPS (50 µg). Twenty-four hours prior to the LPS injection (t=0) and every 24 h thereafter for a period of 4 days, plasma samples were taken after a 4 h-fast. Fibrinogen (A) and E-selectin (B) were measured and the represented values indicate the increase, compared to t=0. Values are represented as means ± SD. n=8, **P*<0.05.

above, and declined ~15% after repeated LPS injections in time (not shown). In all, these data show that LPS transiently increases both the plasma lipid levels and inflammatory response in *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. Whereas the lipid-response after LPS injection was relatively small, LPS injection led to a marked increase of fibrinogen and E-selectin levels. The presence of apoCI had no effect on LPS-induced changes in plasma lipid concentrations, but increased the magnitude of the LPS-induced inflammatory response.

Effect of apoCI on LPS-induced atherosclerosis development

We next examined the effect of the repeated LPS injections on atherosclerosis development, and the effect of apoCI on LPS-induced atherosclerosis. For this purpose, mice were sacrificed 24 h after the tenth LPS injection and the development of atherosclerosis was studied at the level of the aortic root. Representative lesions are shown in Figures 4A-D, and the data of all mice are summarized in Figure 4E. LPS did not affect the atherosclerotic lesion area in *apoe*$^{-/-}$*apoc1*$^{-/-}$ mice (Figure 4A versus 4B), whereas LPS increased the atherosclerotic lesion area by 53% (P<0.05) in *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice (Figure 4C versus 4D). ApoCI expression did not affect atherosclerosis in mice injected with vehicle (Figure 4A versus 4C), yet increased LPS-induced atherosclerosis by 60% (P<0.05) (Figure 4B versus 4D). These data thus indicate that endogenous apoCI expression is a strong determinant for the LPS-induced increase in atherosclerotic lesion size (Figure 4E).

Effect of apoCI on LPS-induced atherosclerotic lesion composition

To study whether LPS treatment affected the composition of the lesions, we characterized the atherosclerotic lesions with respect to monocyte adhesion, the content of CD3+ T-cells, macrophages, SMCs, and collagen. In mice of both genotypes, LPS treatment tended to stimulate monocyte adhesion (Figure 5A) and T-cell recruitment (Figure 5B), albeit the effects did not reach statistical significance. These effects are consistent with previous observations that LPS stimulates monocyte adhesion and T-cell recruitment.[5;18] In mice deficient for apoCI, LPS treatment did not affect the macrophage area (Figure 5C), the SMC area (Figure 5D), and the collagen area (Figure 5E). In contrast, in mice expressing apoCI, LPS increased the macrophage area significantly (P<0.05; Figure 5C), tended to increase SMC area (Figure 5D), and significantly increased collagen area (P<0.05; Figure 5E).
In both the vehicle- and LPS-treated groups, apoCI expression did not affect monocyte adhesion (Figure 5A), yet showed a tendency to increase T-cell recruitment (Figure 5B), and macrophage area (Figure 5C). Furthermore, in the LPS-treated groups, apoCI expression significantly increased SMC (Figure 5D) and collagen area (Figure 5E).

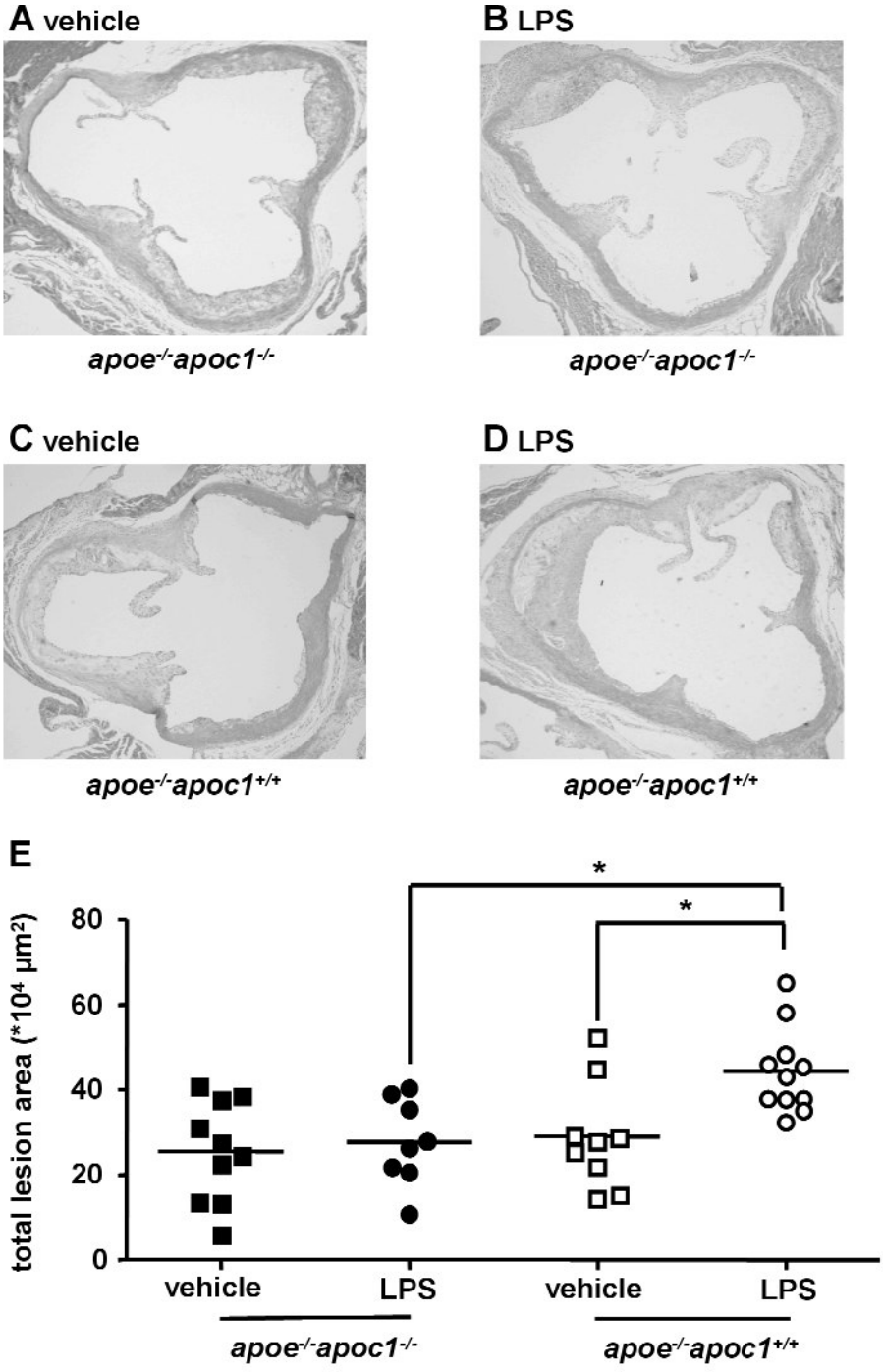

A vehicle
B LPS
apoe-/-apoc1-/-
apoe-/-apoc1-/-
C vehicle
D LPS
apoe-/-apoc1+/+
apoe-/-apoc1+/+
E
total lesion area (*10⁴ µm²)
80
60
40
20
0
*
*
vehicle
LPS
vehicle
LPS
apoe-/-apoc1-/-
apoe-/-apoc1+/+

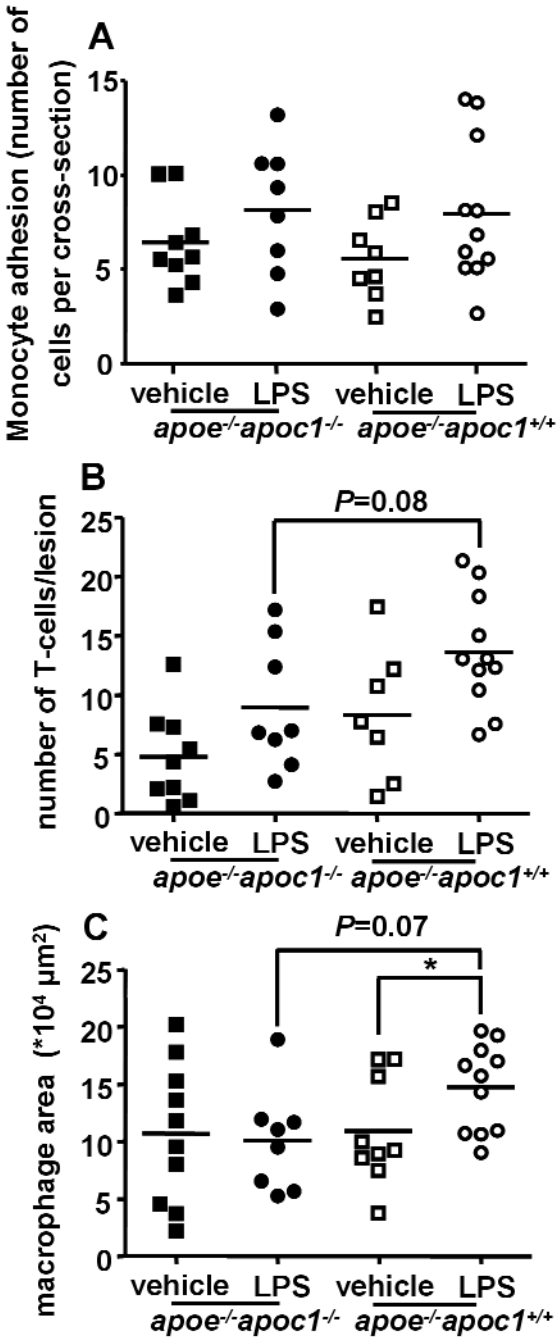
Fig. 4. Effect of apoCI on LPS-induced atherosclerosis development in the aortic root. Twelve week old *apoe*$^{-/-}$*apoc1*$^{-/-}$ (black symbols) and *apoe*$^{-/-}$*apoc1*$^{+/+}$ (white symbols) mice received weekly injections of LPS (50 µg) (circles) or vehicle (squares) for a period of 10 weeks, and were sacrificed after the last injection. Hearts were isolated, cross-sectioned (5 µm) throughout the aortic root, and stained with hematoxylin-phloxin-saffron (HPS). Representative pictures are shown (A-D). Atherosclerotic lesion area was measured in 4 sections per mouse with 40 µm intervals. Each data point represents the mean per mouse (E). n=8-11. **P*<0.05.

Taken together, apoCI expression accelerated atherosclerosis upon LPS treatment. As a result, the atherosclerotic lesions increased in size, and concomitantly contained more SMCs and collagen, reflecting the progression of atherosclerosis.

Discussion

Gram-negative bacteria such as *C. pneumoniae* release LPS upon multiplication or lysis during infections, leading to a chronic inflammatory state that accelerates atherosclerosis in humans and rodents.[9-18] We have shown previously that apoCI binds to LPS, thereby augmenting the inflammatory response to LPS and *K. pneumoniae* in mice *in vivo* and in macrophages *in vitro*.[22] In the present study we investigated the significance of these observations for a chronic inflammatory disease, atherosclerosis, by assessing the effect of apoCI expression on LPS-induced atherosclerosis in *apoe*$^{-/-}$ mice *in vivo*. We found that endogenous apoCI increased atherosclerosis development in *apoe*$^{-/-}$ mice induced by chronic LPS treatment.

We showed that injection of LPS similarly increased the TC-content of IDL/LDL and HDL in both *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice, and marginally increased the TG levels. This response was transient, indicating that the effects were induced after LPS injection and returned to their starting value before the next LPS injection. It is tempting to speculate about the mechanism underlying the lipid changes in our study upon LPS injection. Levels of SAA increased ~500-fold upon LPS injection (results not shown). SAA can replace the major apolipoprotein, apoAI, on HDL, mainly on HDL[3,27] and as such contribute to the increased HDL plasma level, either by stimulating the generation[28] or inhibiting the clearance[29] of HDL.


A
Monocyte adhesion (number of cells per cross-section)
15
10
5
0
vehicle LPS vehicle LPS
apoe-/-apoc1-/- apoe-/-apoc1+/+
B
number of T-cells/lesion
P=0.08
25
20
15
10
5
0
vehicle LPS vehicle LPS
apoe-/-apoc1-/- apoe-/-apoc1+/+
C
macrophage area (*10^4 µm^2)
P=0.07
*
25
20
15
10
5
0
vehicle LPS vehicle LPS
apoe-/-apoc1-/- apoe-/-apoc1+/+

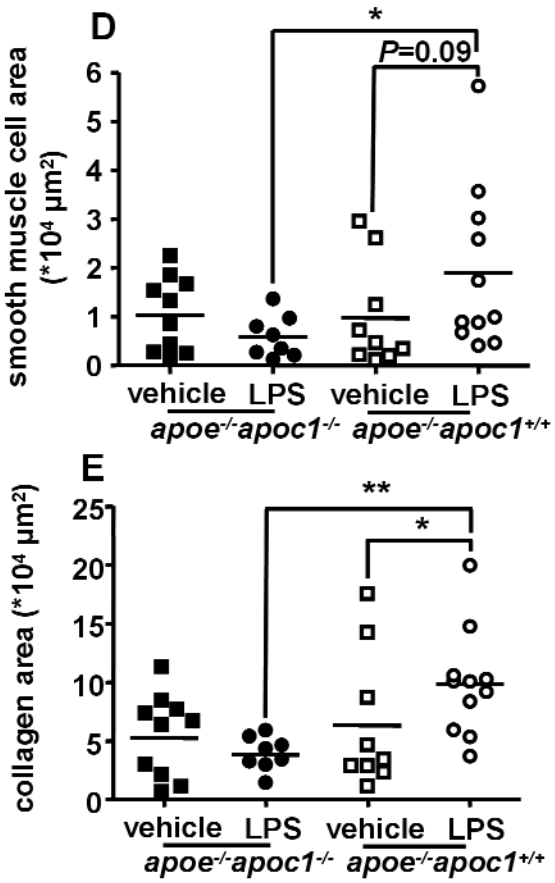


Fig. 5. Effect of apoCI on LPS-induced atherosclerotic lesion composition. In four cross-sections of the aortic root (5 µm), with 40 µm intervals, lesion composition was assessed using immunohistochemistry, with respect to monocyte adhesion (AIA-31240 and hematoxylin positive; A), CD3+ T-cells (CD3 positive; B), macrophages (AIA-31240 positive; C), collagen (Sirius-red positive; D), and smooth muscle cells (α-actin; E). Values are expressed as staining-positive cell number (A, B) or total area (C-E). Each data point represents the mean per mouse. n=8-11. **P*<0.05. ***P*<0.01.

The increased cholesterol content in IDL/LDL might be secondary to reduced lipoprotein lipase (LPL) activity, as LPS injection decreases LPL activity.[30] The effects of LPS on lipid levels were transient and similar in both mouse models, and since the increased levels of plasma cholesterol contributed only ~5% to the total cholesterol exposure over time until atherosclerosis assessment (not shown), it is unlikely that the transient increase in plasma lipid levels after LPS injection is a main contributor to atherosclerosis development.

The augmenting effect of apoCI on LPS-induced atherosclerosis is rather related to a higher inflammatory status in LPS-treated *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice, compared to *apoe*$^{-/-}$*apoc1*$^{-/-}$ mice. We showed that apoCI expression enhanced the effect of LPS on fibrinogen and E-selectin. Fibrinogen is an acute phase protein that is secreted from the liver, reflecting the general inflammatory status of mice, and putatively participating in atherosclerotic lesion development.[31] Fibrinogen is primarily regulated by interleukin (IL)-6.[32] E-selectin is a target gene of the NFκB pathway in macrophages, and reflects the inflammatory state of the vessel wall.[33] ApoCI thus increased the LPS-induced inflammatory responses both systemically and at the vascular level. ApoCI expression *per se* did not affect these inflammatory markers in vehicle-treated mice, an observation which is in agreement with the observed, comparable atherosclerotic lesion area in vehicle-treated *apoe*$^{-/-}$*apoc1*$^{-/-}$ and *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice. We thus conclude that apoCI accelerates the LPS-induced atherosclerosis progression in *apoe*$^{-/-}$ mice mainly as a consequence of increasing inflammation. This is in line with the finding that LPS-treated *apoe*$^{-/-}$ mice expressing human apoAIV show reduced production of pro-inflammatory cytokines and reduced atherosclerosis as compared to their *apoe*$^{-/-}$ littermates.[25]

LPS-treatment did not affect the lesion composition of mice deficient for apoCI, yet increased the macrophage and collagen area in mice expressing apoCI. However, these differences were all neutralized by adjusting for the total lesion area (not shown). SMC and collagen upon apoCI expression in LPS-treated mice remained increased after correction for the total lesion area, yet only significant for collagen (not shown). In fact, comparing lesions from both vehicle groups and the LPS-injected groups with the same atherosclerotic lesion area revealed no differences regarding atherosclerotic lesion composition (not shown). The increased SMC and collagen content were probably mainly the consequence of the more advanced atherosclerosis, where plaques become more enriched in SMCs and collagen.[1-3] Also in advanced lesions we observed adventitial infiltrates of activated lymphocytes, which have been reported to be caused by LPS injections in *apoe*$^{-/-}$ mice.[18] As we only observed these infiltrates in advanced atherosclerotic lesions, we suggest that these infiltrates were rather the consequence of the lesion severity, than caused by LPS specifically. In addition, apoCI been reported to increase apoptosis in human aortic SMC via recruiting neutral sphingomyelinase.[34]

It is unlikely that this effect contributed to atherosclerosis in the aortic root in our study, as apoCI did not affect SMC density in vehicle-treated mice and even increased it in advanced lesions of LPS-treated mice.

At first glance, the finding that apoCI expression did not significantly affect atherosclerosis in the vehicle-treated mice seems to be in contrast with our previous data showing that apoCI accelerates atherosclerosis as related to increased VLDL-lipid levels in mice of 26 weeks of age.[35] However, in the present study, VLDL-lipid levels were somewhat increased upon apoCI expression, yet the absolute values in both mouse genotypes were ~20% lower than in our previous study. In fact, the relatively low lipid levels in our present study led to a relatively slow progression of atherosclerosis which was not different between mice of both genotypes, and enabled us to investigate the effect of apoCI on LPS-induced atherosclerosis specifically. Indeed, mice need to express a certain level of hyperlipidemia for LPS to be effective on atherosclerosis development, as was demonstrated in former studies using *C. pneumoniae* that mainly exerts its effects via LPS.[15;36;37] We speculate that the effects of apoCI on LPS-induced atherosclerosis are mediated via TLR4, since TLR4 is primarily activated by LPS[38] and has been demonstrated to be involved in LPS-induced atherosclerosis development in mice.[16] The mechanism of the apoCI-induced LPS response is currently under investigation.

We conclude that apoCI is crucially involved in the LPS-induced atherosclerosis in *apoe*$^{-/-}$ mice, mainly as a consequence of enhancing the inflammatory response. We anticipate that in humans that suffer from chronic inflammation, plasma apoCI may enhance atherosclerosis development and CVD.

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