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Factor XI Deficiency Protects Against Atherogenesis in Apolipoprotein E/Factor XI Double Knockout Mice

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Abstract

Objective—Atherosclerosis and atherothrombosis are still major causes of mortality in the Western world, even after the widespread use of cholesterol-lowering medications. Recently, an association between local thrombin generation and atherosclerotic burden has been reported. Here, we studied the role of factor XI (FXI) deficiency in the process of atherosclerosis in mice.

Approach and Results—Apolipoprotein E/FXI double knockout mice, created for the first time in our laboratory. There was no difference in cholesterol levels or lipoprotein profiles between apolipoprotein E knockout and double knockout mice. Nevertheless, in 24-week-old double knockout mice, the atherosclerotic lesion area in the aortic sinus was reduced by 32% (P=0.004) in comparison with apolipoprotein E knockout mice. In 42-week-old double knockout mice, FXI deficiency inhibited atherosclerosis progression significantly in the aortic sinus (25% reduction, P=0.024) and in the aortic arch (49% reduction, P=0.028), with a prominent reduction of macrophage infiltration in the atherosclerotic lesions.

Conclusions—FXI deprivation was shown to slow down atherogenesis in mice. The results suggest that the development of atherosclerosis can be prevented by targeting FXI.

Keywords

atherosclerosis; factor XI

Atherosclerosis is a major cause of morbidity and mortality in developed countries.[1] It is a chronic inflammatory disease of the arterial wall and is characterized by lipid deposition, leukocyte infiltration, and smooth muscle proliferation. Atherothrombosis occurs as a

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Disclosures

None.

consequence of the production of a thrombus on a rupture or erosive atherosclerotic plaque, resulting in myocardial infarction or ischemic stroke.[2]

The role of coagulation factors in atherosclerosis is not clear. Atherosclerotic plaques contain thrombogenic materials[3] and coagulation factors,[4] which conceivably serve as a shield against vascular injury.[5] Remarkably, an association between local thrombin generation and atherosclerotic plaque burden has been reported.[5] One of the mechanisms by which thrombin may be involved in the process of atherogenesis is the activation of protease-activated receptors 1 and 4. In addition, thrombin's cleavage of the complementary proteins C3 and C5 into their active forms, which leads to plaque instability and atherothrombosis, is implicated in the process of intraplaque inflammation with other local coagulation factors.[5] In contrast to atherothrombosis, in which the role of coagulation factors is evident, the association between coagulation factors and atherosclerosis per se remains unclear. It has been suggested that in mice, the perishing of thrombomodulin, which is the result of endothelial dysfunction or apoptosis during the process of atherogenesis, leads to the potentiating of atherosclerosis through reduced protein C activity and increased thrombin generation.[6] In addition, apolipoprotein E (apoE) knockout mice with tissue factor pathway inhibitor deficiency exhibited a greater atherosclerotic burden, indicating that tissue factor pathway may have a role in the process of atherogenesis.[7] However, reduced tissue factor in apoE knockout mice did not affect the development of atherosclerotic lesions in mice.[8]

Interestingly, greater inhibition of atherogenesis was observed in apoE knockout mice with FVIII deficiency in comparison with apoE knockout mice.[9,10] Moreover, apoE knockout mice treated with factor Xa inhibitors showed reduced plaque burden.[11] In human, rivaroxaban, a direct oral FXa inhibitor has been shown to reduce significantly myocardial infarction and stroke.[12] Taken together, this evidence suggests that diverse coagulation factors contribute to both atherosclerosis and atherothrombosis.

The involvement of coagulation factors in the process of atherogenesis[2] might lead one to conclude that hemophilic patients have reduced atherosclerotic burden.[13] However, although some clinical trials have shown a reduced incidence of myocardial infarction in patients with hemophilia A,[14,15] others have shown that the degree of atherosclerosis burden, as measured by different modalities such as intima–media thickness, degree of coronary artery calcification, plaque density of large vessels, and mode of endothelial vascular dilatation, is similar to that in the general population.[16,17] Nonetheless, reduced cardiovascular mortality[16,18] has been shown in hemophilic patients and is probably because of reduced fatal occlusive atherothrombosis.[15]

The role of factor XI (FXI) in atherosclerosis is ambiguous. Notably, high levels of FXI are associated with increased risk of stroke and associated marginally with myocardial infarction.[19,20] However, in patients with severe FXI deficiency, a reduced incidence of ischemic stroke but not of myocardial infarction was observed.[21,22] In animal models, it was shown that deprivation of FXI did protect against injury-induced thrombosis[23,24] and that apoE knockout mice treated with FXI antisense oligonucleotides were protected from thrombosis on acutely ruptured atherosclerotic plaques.[25] However, whether FXI is involved

in atherogenesis has not been investigated, despite its involvement in hemostasis and suggested role in modulating inflammation.[26,27] Therefore, this study investigated whether lack of FXI affects the process of atherogenesis in apoE/FXI double knockout (DKO) mice.

Materials and Methods

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

Results

To investigate the effect of FXI deficiency on atherogenesis, we cross-bred the well-known model for atherosclerosis, apoE knockout mice, with FXI knockout mice and generated an atherosclerosis mouse model, apoE/FXI DKO, with severe FXI deficiency.

Phenotype Characterization of apoE/FXI DKO Mice

FXI deficiency in the background of apoE knockout did not affect fertility and mouse growth (data not shown). FXI deficiency was validated by measuring FXI activity in plasma, and FXI protein expression was assayed by Western blot. In C57Bl/6 and apoE knockout mice, a clear band of FXI (75 kD) was detected, whereas in both FXI knockout and apoE/FXI DKO mice, no FXI was detected (Figure 1A).

FXI activity was measured according to activated partial thromboplastin time using FXI deficient human plasma. The apparent activity of FXI was significantly reduced (<10%) in both FXI knockout and apoE/FXI DKO, compared with C57BL/6. Unexpectedly, the activity of FXI was also reduced in apoE knockout compared with C57BL/6 by about 50%. Nonetheless, the activity of FXI was significantly lower in apoE/FXI DKO compared with apoE knockout alone, and in contrast to apoE species, no FXI was detected by Western blot (Figure 1A). Notably, in the heterozygote mice, apoE knockout/ $FXI^{+/-}$ with a single copy of FXI, the activity of FXI was similar to that in apoE knockout mice (Figure 1B).

Effect of FXI Deficiency on Plasma Lipid Levels and Atherogenesis

Atherosclerosis was assessed in apoE/FXI DKO in 24-week-old (representing early atherosclerosis) and 42-week-old (representing advanced atherosclerosis) mice and compared with apoE knockout mice. Four groups of mice were included in both the first and the second experiments—C57BL/6, FXI knockout, apoE knockout, and apoE/FXI DKO and a fifth group, apoE knockout/$FXI^{+/-}$, was added in the second experiment.

As expected, plasma cholesterol levels were higher in apoE knockout and apoE/FXI DKO mice compared with C57BL/6 and FXI knockout mice (Figure 2A). FXI deficiency did not affect plasma cholesterol levels, and the cholesterol levels in apoE/FXI DKO were similar to those in apoE knockout mice. In the 2 sets of experiments, plasma triglyceride levels were lower in apoE/FXI DKO at 24 weeks. However, no correlation between triglyceride and the size of atherosclerotic lesions at 24 weeks of age was observed (Pearson correlation

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coefficient −0.009, *P*=0.674). Interestingly, at the advanced age of 42 weeks, plasma triglyceride levels were similar (Figure 2B). Fast protein liquid chromatography analysis showed that cholesterol distribution in plasma lipoproteins was not affected by FXI deficiency (Figure 2C). As with total plasma triglyceride, the levels of triglyceride in very low–density lipoprotein were lower in apoE/FXI DKO mice at 24 weeks (data not shown) of age and similar in 42-week-old mice.

We determined the effect of FXI deficiency on the atherosclerotic lesion area in the aortic sinus in 24-week-old mice and in the aortic sinus and aorta in 42-week-old mice. In 24-week-old mice, the atherosclerotic lesion area was reduced by 32% (*P*=0.004) in apoE/FXI DKO compared with control apoE knockout mice (Figure 3A and 3C). As expected, C57BL/6 and FXI knockout did not develop atherosclerosis at all. In 42-week-old mice, apoE knockout developed advanced lesions, and as in the younger mice, FXI deficiency inhibited atherogenesis progression significantly in the aortic sinus (25% reduction, *P*=0.024; Figure 3B and 3D) and in the aortic arch (49% reduction, *P*=0.028; Figure 4A and 4B). Hence, FXI deficiency resulted in inhibition of atherogenesis in both early and advanced age without having an effect on plasma cholesterol levels.

To examine the effect of FXI deficiency on the stability of the atherosclerotic lesion, we evaluated collagen and macrophage content in the plaques. Collagen was observed across the entire thickness of the neointima in both apoE knockout and apoE/FXI DKO mice (Figure 5). Because FXI deficiency was previously shown to reduce inflammation in mouse models, we performed an assay to determine whether FXI deficiency reduces the number of macrophages in the atherosclerotic plaques. Immunohistochemistry staining of macrophages with anti-CD68 antibody showed that the number of macrophages in apoE/FXI DKO in lesions of 42-week-old mice was significantly reduced compared with apoE knockout mice (Figure 6), indicating reduced inflammation.

Discussion

This is the first study to investigate the role of FXI in the induction and development of atherosclerosis,[28] despite the fact that coagulation factors including FXI have been found inside atherosclerotic plaques and a link between coagulation and atherogenesis has been reported.[4] Therefore, we investigated whether FXI promotes atherogenesis by using an apoE/FXI DKO mouse model, created for this purpose in our laboratory. A significant reduction of both early and advanced atherosclerotic lesions was observed in the aortic sinus and aortic arch in the DKO mice, suggesting that FXI accelerates atherogenesis.

The first objective of this study was to create DKO mice, deficient in both apoE and FXI. In contrast to the well-known $LDLR^{-/-}$ mouse model that develops atherosclerosis only when fed an atherogenic diet, $apoE^{-/-}$ mice develop atherosclerosis on both a regular chow diet and a high-fat diet.[29] Therefore, the apoE/FXI DKO mouse model created in this study enables the investigation of atherogenesis under these 2 diet regimes. Although C57BL6 mice, the background of $FXI^{-/-}$ mice, develop fatty streaks when fed an atherogenic diet,[30] the effect of FXI deficiency on fatty streak development, the hallmark of early stages of atherosclerosis, has not been studied. The newly created DKO mice appeared normal and

were fertile, with normal litter sizes. About coagulation, FXI has not been found in DKO mice, and its activity is similar to that in FXI knockout mice, that is, <10% of control mice. We also measured FXI activity in heterozygote mice, apoE knockout/FXI$^{+/-}$, and showed that a single copy of FXI resulted in 50% of activity compared with control mice. Nonetheless, the reduced activity in the heterozygote mice did not affect atherogenesis. As such, the fact that apoE species had also a prolonged activated partial thromboplastin time as previously been reported[31] should not affect our results. Notably, FXI deficiency had no effect on fasting plasma cholesterol and triglyceride levels, and their distributions between lipoproteins were similar to the distribution in apoE knockout mice. These findings indicate that the inhibition effect on atherogenesis is not because of the lipid-lowering effect of FXI deficiency.

Because the levels of plasma cholesterol and lipoproteins in the DKO mice were similar to those of apoE knockout mice, the DKO mouse seems to us a reasonable model for studying the effect of FXI on atherogenesis through other mechanisms. In this study, we aimed, first, to investigate our hypothesis that FXI deficiency would inhibit atherogenesis and, second, to gather preliminary data about its role in inflammation in the process of atherogenesis. As expected, complete FXI deficiency led to significant inhibition of atherosclerosis in both early and advanced stages in the DKO model (Figures 3 and 4) compared with apoE knockout mice. Because there are many tissue factors inside the atherosclerotic plaque that can potentiate coagulation,[32] we wonder whether the effect of FXI deficiency on atherogenesis is related to coagulation at all. Several findings have implicated FXI in inflammation: thrombi formed on the ruptured plaques, from perturbation carotid arteries of FXI antisense oligonucleotides-treated mice shows lower infiltration of macrophages[25]; FXI antibody 14E11 inhibits the course of bowel perforation–induced peritoneal sepsis in mice[26,27]; and display reduced inflammation during listeriosis.[33] These findings point to the critical role of inflammation in the process of atherosclerotic lesion development[34] and motivated us to measure macrophage content in the atherosclerotic lesions in the advanced stage, as an indicator of the inflammatory index. In line with the studies described above, we also found that the absence of FXI leads to reduced inflammation inside the advanced atherosclerotic plaques of 42-week-old DKO mice. Interestingly, reduced macrophages in plaques were also reported in apoE knockout mice with hypoprothrombinemia.[35] In future studies, we will further characterize the effect of FXI on atherosclerosis and investigate the mechanisms of its effect on inflammation. Nevertheless, these findings may indicate that lack of FXI inhibits atherogenesis by reducing inflammation inside the atherosclerotic plaque.

The results obtained in the DKO model suggest that along with the use of cholesterol-lowering drugs, targeting FXI may help to prevent or retard the development of atherosclerosis and atherothrombosis in humans. It should be noted that FXI plays a minor role in sustaining hemostasis[36] and that it has recently been shown that treatment directed at this protein using FXI antisense oligonucleotide was not associated with a risk of major bleeding.[37] In contrast to targeting FXI, targeting other coagulation factors such as FVIII is less attractive because of the increased risk of spontaneous bleeding. Targeting factor XII can compromise the stability of arterial thrombi and increase the risk of embolization. This

may explain why patients with reduced FXII are at increased risk for cardiovascular disease.[38]

It may well be that one of the reasons patients with hemophilia are not protected from atherosclerosis is that those patients currently undergo replacement therapy, unlike in FXI deficiency that prophylactic treatment is administered for only major procedures.

Our results should be interpreted with caution before they are generalized to humans, because in mice atherosclerotic lesions are histologically different and ruptured atherosclerotic plaques is uncommon. The data suggest that treatment with FXI inhibitor could inhibit the process of atherogenesis, thus improving survival.

Materials and Methods

Animal models

FXI deficient mice (FXI KO) on a C57BL/6 background provided by Prof. D Gailiani (Vanderbilt University) were crossbred with apoE KO mice (Jackson Laboratory, USA) carrying the same background. The presence of apoE/FXI DKO was verified by PCR analysis using DNA extracted from tail clip samples. For apoE, the oligonucleotide (sigma) 5'-GGCTAGCCGAGGGAGAGCCG served as a common primer for the wild-type (WT) and KO alleles. The primers for the WT and KO alleles were 5'-TGTGACTTGGGAGCTCTGCAGC and 5'-GCCGCCCCGACTGCATCT, respectively. For FXI, the oligonucleotide (Metabion) 5'-AGGCTTATATCCAGCGGCTCGGACTC served as a common primer for the WT and KO sequence. The primers for the WT and KO alleles were 5'-TTGCAGCAAAGATGAGTACGTGAACC and 5'-TTCCTGACTAGGGGAGGAGTAGAAGGTG, respectively. The presence of FXI in plasma was demonstrated by western blot. Briefly, 3 µl of plasma per lane were loaded and separated on an SDS-PAGE 7.5% gel. The proteins migrated to the nitrocellulose membrane, and after blocking with 5% milk in TBS-T buffer (20 mM Tris base, 150 mM NaCl, 0.1% sodium azide, 3% BSA, and 0.1% Tween 20), the membranes were probed with biotinylated 14E11 primary antibody (5 µg/ml; provided by Prof. D Gailiani), which selectively inhibits the activation of FXI by factor XIIa (Tucker Blood 2012). Detection was performed with Streptavidin-Horseradish peroxidase (HRP; 1:200 dilution, R&D System) and chemiluminescence.

Phenotype characterization

After overnight fasting, blood was drawn from the retro-orbital sinus in tubes with 0.11 M sodium citrate or 10% EDTA for plasma preparation.

FXI activity was determined by an assay based on aPTT, using a coagulation analyzer (ACL-TOP 500). Calibration with a pool of mouse plasma was performed similarly to calibration with human plasma (data not shown). Therefore, in all experiments, calibration was performed using human plasma (Instrumentation Laboratory).

Cholesterol and TG determination: Colorimetric enzymatic procedures were used to measure plasma total cholesterol and triglycerides (Roche/Hitachi).

Lipoprotein profile: Plasma lipoproteins were separated by size exclusion chromatography using a superose-6 column (1 × 30 cm) on an FPLC system (Pharmacia), as described previously.[1] Briefly, a 200 μL aliquot of pooled plasma from each experimental group was injected into the column and separated with a buffer containing 0.15 mmol/L NaCl, 0.01 mmol/L $NaHPO_4$, and 0.1 mmol/L EDTA, pH 7.5, at a flow rate of 0.5 mL/min.

Study design

In all experiments, 12-week-old male mice were housed in plastic cages under a 12:12 hour light/dark cycle with a chow diet and water provided ad libitum throughout the experiment.

The first experiment comprised four groups: (i) C57BL/6 (Harlan, Israel; n = 8), (ii) FXI KO (n = 12), (iii) apoE KO (n = 14), and (iv) apoE/FXI DKO (n = 8). At 24 weeks of age, mice were killed and analyzed for early atherosclerosis. The second experiment comprised five groups: (i) C57BL/6 (Harlan, Israel; n = 6), (ii) FXI KO (n = 14), (iii) apoE KO (n = 20), (iv) apoE/FXI DKO (n = 21), similar to the fourth group in the first experiment, and (v) apoEFXI+/− (n = 8), an additional group with FXI deficiency in one allele. Mice were killed at 42 weeks of age and analyzed for advanced atherosclerosis.

The Animal Care and Use Committee of Sheba Medical Center, Tel-Hashomer, approved all animal protocols (approval numbers: 773/12, 896/14).

Atherosclerosis assessment

Quantification of atherosclerotic lesions was performed by calculating the lesion size in two distinct anatomical regions: the aortic sinus, with Oil Red O staining, and the aortic arch, with Sudan IV staining. The heart and upper section of the aorta were removed and embedded in optimal cutting temperature compound (Miles Inc.). Throughout the aortic sinus, 10 μm thick sections were taken for analysis.[2] The distal portion of the aortic sinus was identified by the presence of the aortic valve, located between the left ventricular outflow tract and the ascending aorta. Sections (10 μm thick) were evaluated for atherosclerotic lesions after staining with Oil Red O. For aortic arch assessment, the aorta was removed from the aortic arch to the iliac branches and fixed in 4% formalin. The aorta was cut longitudinally and stained with Sudan IV (Fluka). Lesion-area analysis was performed by blindly using NIS Elements Imaging Software (Nikon).

Atherosclerosis characterization

We performed immunohistochemical staining on sections (5 μm thick) derived from the aortic sinus. The presence of macrophages in the lesion was evaluated using the anti-CD68 antibody (Abcam, Cambridge, UK). For macrophage evaluation, each section was graded on a scale of 0–4, where 0 represents sections without or with low macrophage concentration and 4 represents the highest macrophage concentration.

Collagen fibers within lesions were identified with Masson’s trichrome staining. These analyses were performed in a blinded fashion by an expert pathologist (GS).

Statistical analyses

One-way ANOVA was used to compare the mouse group's effect on atherogenesis, and the post hoc Tukey method was used for multiple pairwise comparisons. Student's *t*-test was used to compare the two independent values. Correlation between two groups was examined by Pearson's chi-squared test. Significance was set at $p < 0.05$. Values are presented as means ± SE. All statistical analyses were conducted using SPSS version 12.0 (SPSS Inc.).

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Nonstandard Abbreviations and Acronyms

apoE	apolipoprotein E
DKO	double knockout
FXI	factor XI

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Significance

Here, we showed that knockout factor XI gene retards early and advanced atherosclerosis in apolipoprotein E knockout mice. This was associated with a significant reduction in macrophage infiltration in the atherosclerotic plaque of apolipoprotein E/factor XI double knockout mice in comparison with apolipoprotein E knockout mice. This work encourages to study whether targeting factor XI delays the process of atherosclerosis in humans.

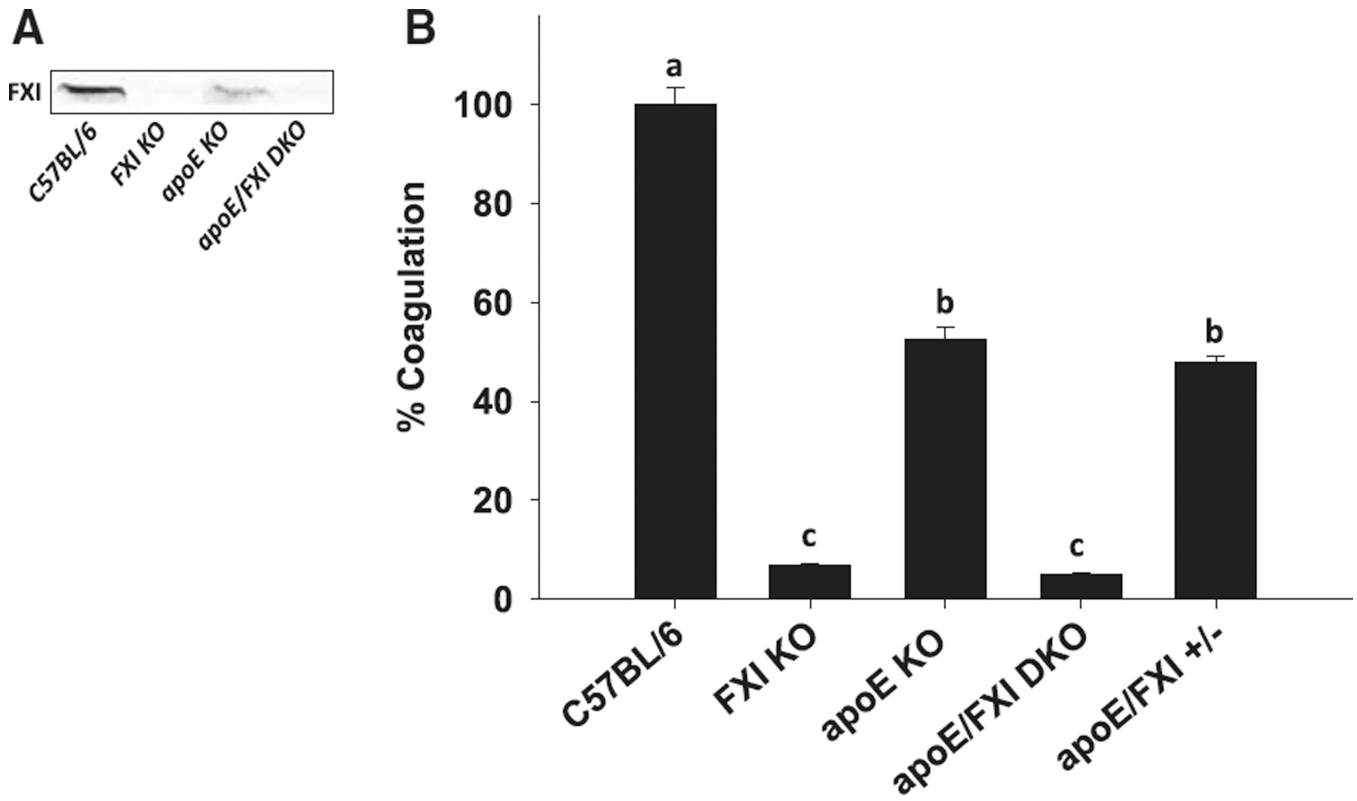

Figure 1.

Phenotype characterization of mice. **A**, Factor XI (FXI) detected by Western blot in plasma of C57BL/6, FXI knockout (KO), apolipoprotein E (apoE) KO, and apoE/FXI double KO (DKO) mice using biotinylated 14E11 antibody. **B**, FXI activity in plasma of C57BL/6 (n=7), FXI KO (n=10), apoE KO (n=14), apoE/FXI DKO (n=12), and apoE/FXI$^{+/-}$ (n=5) mice according to activated partial thromboplastin time–based assay.

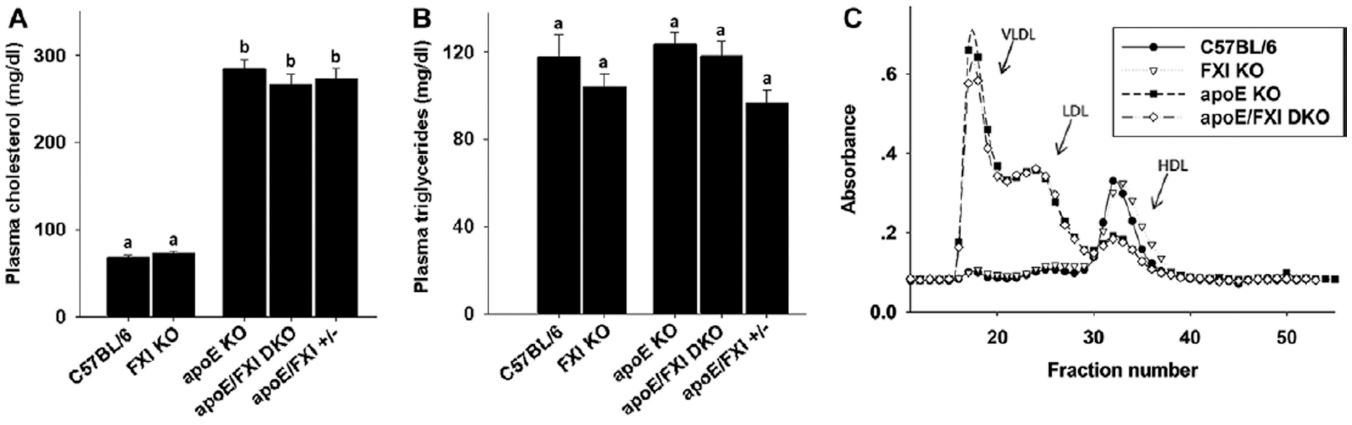

Figure 2.

Plasma lipid levels. **A**, Plasma cholesterol levels of C57BL/6 (n=6), factor XI knockout (FXI KO; n=12), apolipoprotein E knockout (apoE KO; n=20), apoE/FXI double KO (DKO; n=20), and apoE/FXI$^{+/-}$ (n=8) 42-week-old mice. **B**, Plasma triglyceride levels of C57BL/6 (n=6), FXI KO (n=14), apoE KO (n=20) apoE/FXI DKO (n=19), and apoE/FXI$^{+/-}$ (n=8) 42-week-old mice. **C**, Cholesterol distribution in the very low–density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) of C57BL/6, FXI KO, apoE KO, and apoE/FXI DKO 42-week-old mice as measured by fast performance liquid chromatography. Pooled plasma from each experimental group was assessed. Each point represents the mean of 2 separation measurements. Bars are mean±SE; means without a common letter differ; $P<0.05$.

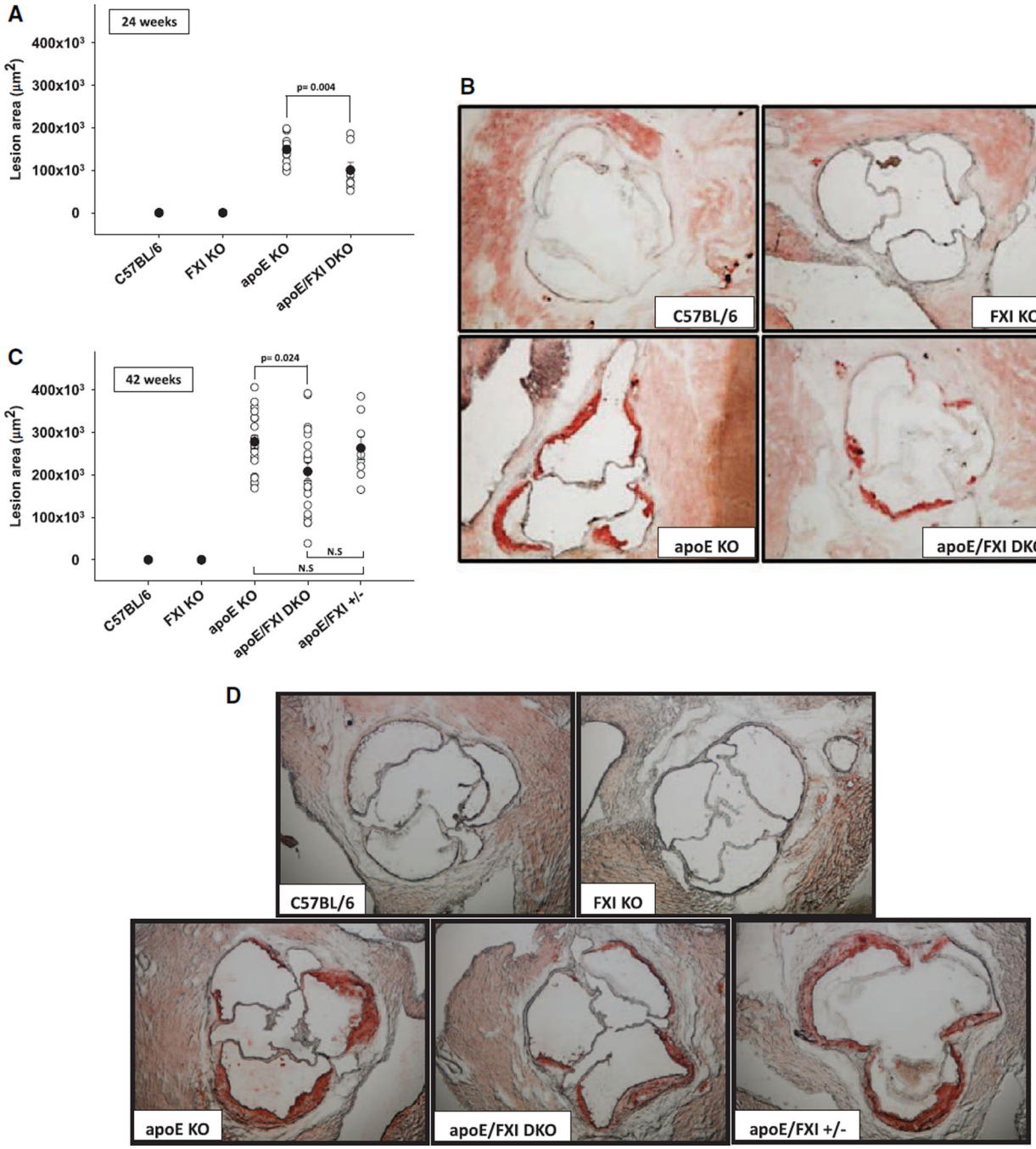

Figure 3.

Aortic sinus atherosclerotic lesion area of 24- and 42-week-old mice. **A**, Lesion area of 24-week-old C57BL/6 (n=8), factor XI knockout (FXI KO; n=12), apolipoprotein E knockout (apoE KO; n=14), and apoE/FXI double KO (DKO; n=8) mice and (**B**) 42-week-old C57BL/6 (n=6), FXI KO (n=14), apoE KO (n=20), apoE/FXI DKO (n=21), and apoE/FXI$^{+/-}$ (n=8) mice. Atherosclerotic lesion area was quantified after staining with Oil Red O. Empty circles represent mean of 3 sections per mouse. Black circles represent group mean ±SE. Representative aortic sinus lesions of each group of (**C**) 24-week-old mice: C57BL/6,

FXI KO, apoE KO, and apoE/FXI DKO and (**D**) 42-week-old mice: C57BL/6, FXI KO, apoE KO, apoE/FXI DKO, and apoE/FXI$^{+/-}$ (magnification ×40). N.S. indicates not significant.

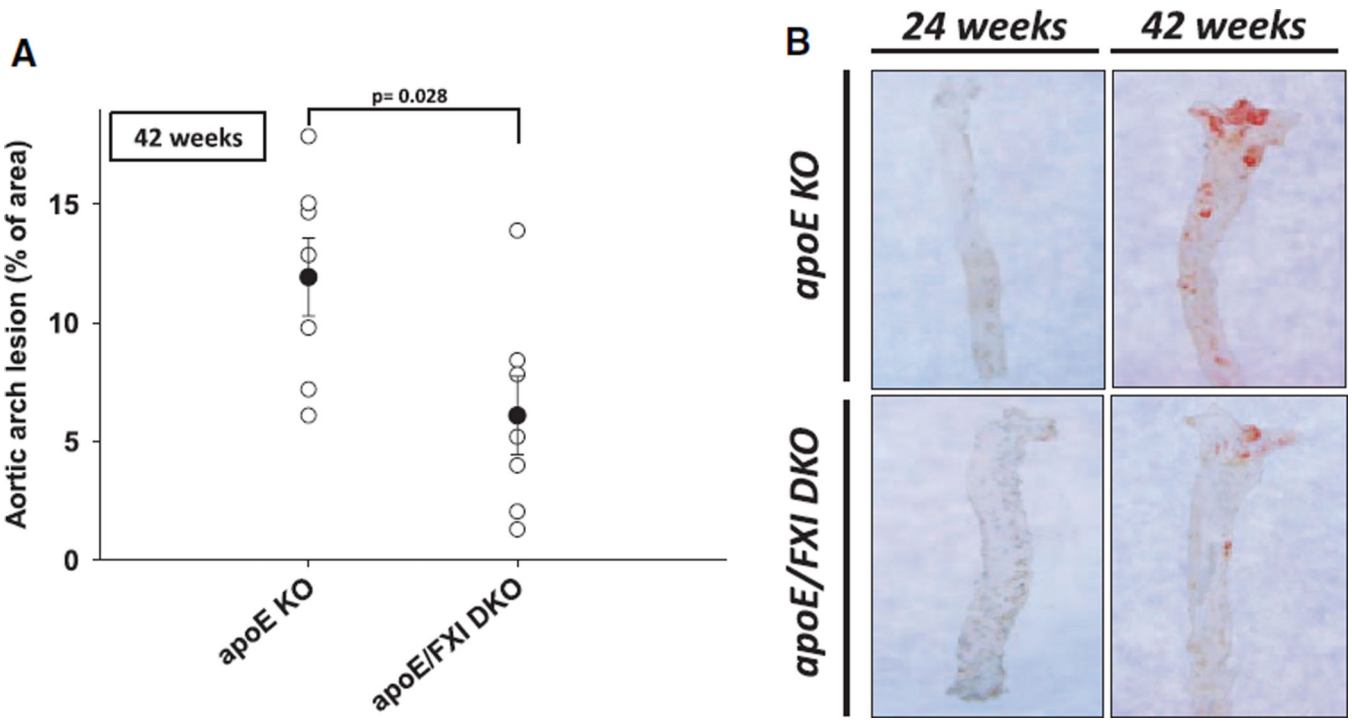

Figure 4.

Atherosclerosis assessment of the aortic arch in 42-week-old mice. **A**, Atherosclerotic lesion area of apolipoprotein E knockout (apoE KO) mice (n=7) compared with apoE/factor XI double KO (FXI DKO) mice (n=7) at 42 weeks of age. The atherosclerotic lesion area was quantified by Sudan IV staining. Empty circles represent mean area of 3 sections per mouse. Black circles represent group mean±SE. **B**, Representative photographs of aortic arch of 24- and 42-week-old mice.

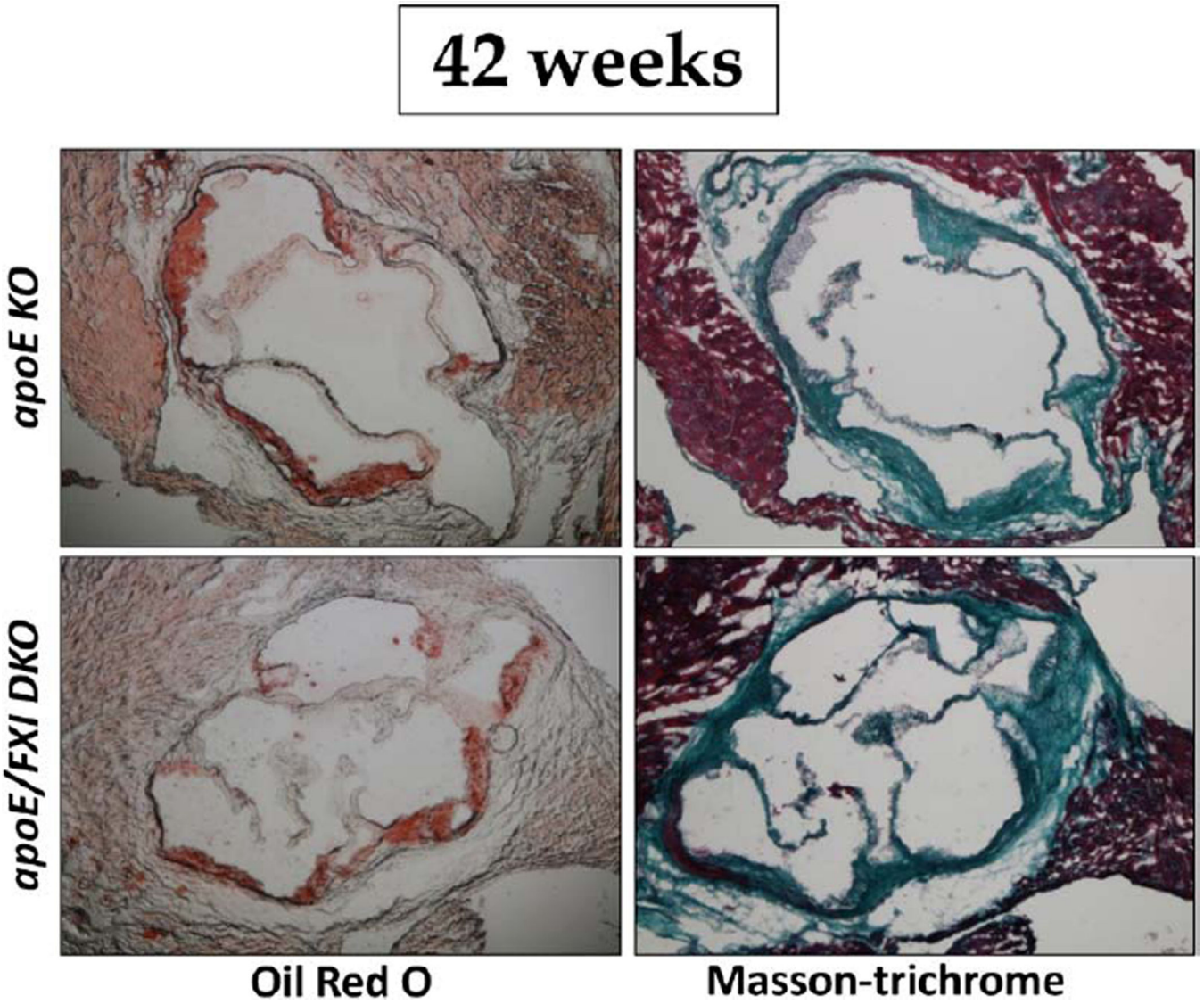

Figure 5.

Collagen content in the apolipoprotein E (apoE)/factor XI double knockout (FXI DKO) mice. Atherosclerotic lesion area was quantified after staining with Oil Red O and collagen content by Masson-trichrome staining in apoE KO (n=8) and apoE/FXIDKO (n=8).

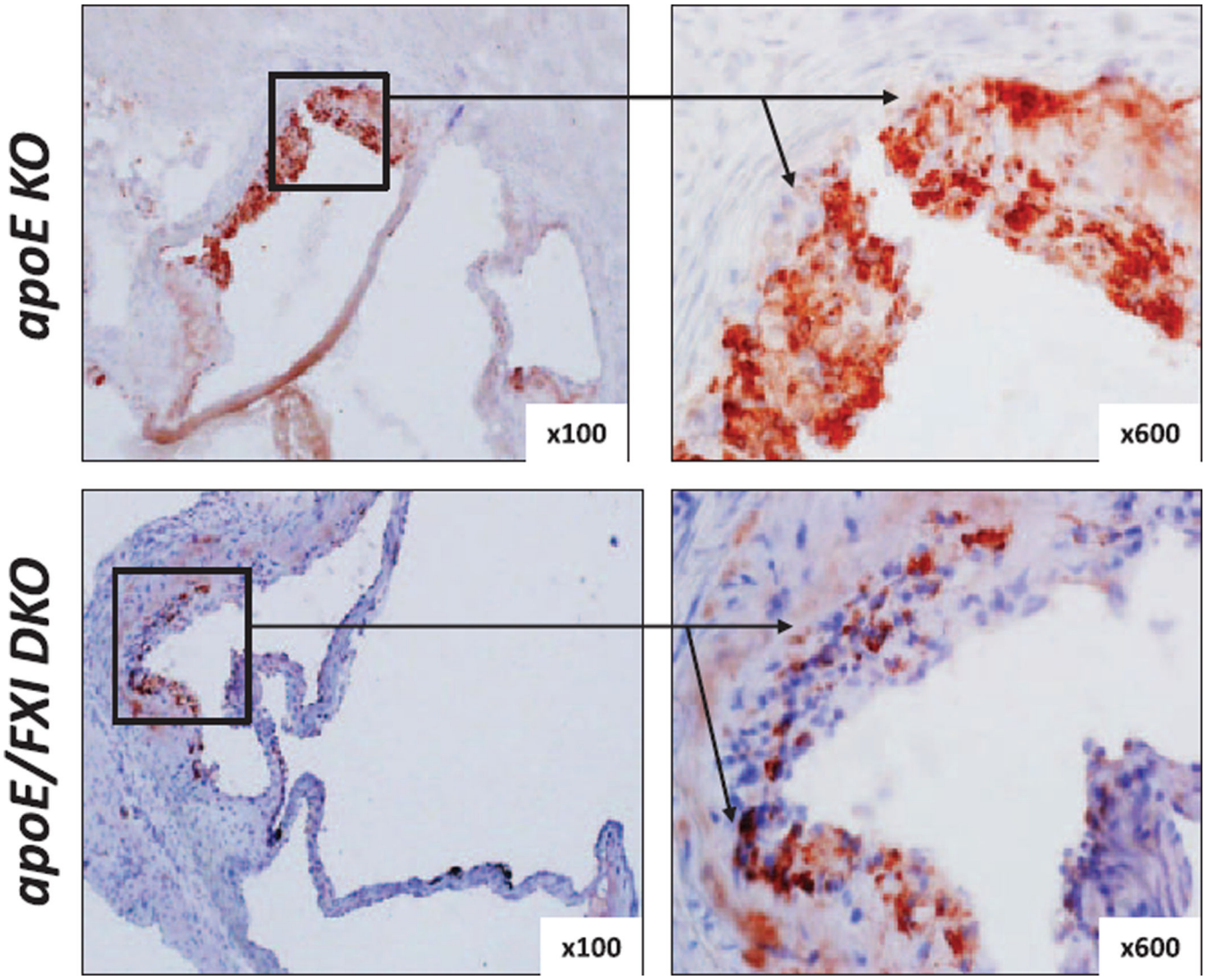

Figure 6.

Macrophage infiltration in aortic sinus atherosclerotic lesions in apolipoprotein E (apoE) and apoE/factor XI double knockout (FXI DKO) 42-week-old mice. Macrophage infiltration was assessed by anti-CD68 antibody.