Fibronectin Splicing Variants Containing Extra Domain A Promote Atherosclerosis in Mice Through Toll-Like Receptor 4

Prakash Doddapattar,* Chintan Gandhi,* Prem Prakash, Nirav Dhanesha, Isabella M. Grumbach, Michael E. Dailey, Steven R. Lentz, Anil K. Chauhan

Objective—Cellular fibronectin containing extra domain A (EDA-FN) is abundant in the arteries of patients with atherosclerosis. Several in vitro studies suggest that EDA-FN interacts with Toll-like receptor 4 (TLR4). We tested the hypothesis that EDA-FN exacerbates atherosclerosis through TLR4 in a clinically relevant model of atherosclerosis, the apolipoprotein E-deficient (ApoE−) mouse.

Approach and Results—The extent of atherosclerosis was evaluated in male and female EDA+/ApoE− mice (which lack EDA−FN), EDA+/ApoE− mice (which constitutively express EDA−FN), and control ApoE− mice fed a high-fat Western diet for 14 weeks. Irrespective of sex, EDA+/ApoE− mice exhibited a 2-fold increase in atherosclerotic lesions (aorta and aortic sinus) and macrophage content within plaques, whereas EDA+/ApoE− mice exhibited reduced atherosclerotic lesions (P<0.05 versus ApoE−, n=10–12 mice/group), although cholesterol and triglyceride levels and circulating leukocytes were similar. Genetic ablation of TLR4 partially reversed atherosclerosis exacerbation in EDA+/ApoE− mice (P<0.05) but had no effect on atherosclerotic lesions in EDA+/ApoE− mice. Purified cellular FN, which contains EDA, potentiated dose-dependent NF-kB-mediated inflammation (increased phospho-NFκB p65/NFκB p65, tumor necrosis factor-α, and interleukin-1β) in bone marrow–derived macrophages from EDA+/ApoE− mice but not from EDA+/TLR4+/ApoE− mice. Finally, using immunohistochemistry, we provide evidence for the first time that EDA-FN colocalizes with macrophage TLR4 in murine aortic lesions and human coronary artery atherosclerotic plaques.

Conclusions—Our findings reveal that TLR4 signaling contributes to EDA−FN–mediated exacerbation of atherosclerosis. We suggest that EDA−FN could be a therapeutic target in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2015;35:2391-2400. DOI: 10.1161/ATVBAHA.115.306474.)

Key Words: apolipoprotein E ■ atherosclerosis ■ cellular fibronectin EDA ■ macrophages ■ TLR4

Atherosclerosis is a chronic inflammatory disease that affects large arteries and is characterized by plaques composed of lipids, calcium, extracellular matrix (ECM), and inflammatory cells, including monocytes/macrophages, T lymphocytes, and neutrophils. Much of the morbidity and mortality associated with atherosclerosis is as a result of coronary artery plaques, which cause luminal obstruction because of vessel stenosis and occlusive thrombus triggered by rupture of unstable plaques. During progression of atherosclerosis, extensive remodeling of ECM takes place. Several studies have found that altered ECM participates at different stages of atherosclerosis. There is emerging evidence that in chronically inflamed tissues, aberrant expression of ECM proteins or ECM fragments can modulate migration of leukocytes and immune cell responses at these sites. In healthy human arteries, the endothelium resides on an ECM that is mainly composed of collagen type IV and laminin. In contrast, the ECM of atherosclerotic arteries contains abundant deposits of fibronectin (FN) in both humans and murine models, suggesting a functional role for FN in the pathophysiology of atherosclerosis.

FN is a dimeric glycoprotein that is known to play an important role in several cellular processes. FN exhibits diversity at the protein level as a consequence of alternative splicing of a single primary transcript at 3 exons that encode the extra domain A (EDA), the extra domain B, and the Type III homologies connecting segment. The major isoform of FN found in plasma lacks both the alternatively spliced EDA and extra domain B segments and is synthesized by hepatocytes. The predominant isoform of FN found in the ECM, which is...
known as cellular FN (cFN), contains the EDA or extra domain A segments or both. cFN in the ECM is synthesized by vascular cells, including endothelial and vascular muscle cells. The amino acid sequence of the EDA is highly conserved (>90%) in mammals, as is its splicing pattern, with either total or partial inclusion of the EDA exon in FN gene by alternative splicing.

Inclusion of the EDA exon in FN gene by alternative splicing is specifically regulated during several biological and pathological processes, including cutaneous wound healing, vascular intimal proliferation, vascular hypertension, cardiac transplantation, and fibrosis of the lung, liver, and kidney, suggesting a functional role for cFN containing EDA in these pathological processes.

EDA+-FN are specifically expressed in atherosclerotic but not healthy arteries, suggesting a possible functional role for EDA+-FN in atherosclerosis. Two prior studies have found that deletion of the EDA exon of the FN gene reduces atherosclerosis, and pathological processes, including cutaneous wound healing, vascular intimal proliferation, vascular hypertension, cardiac transplantation, and fibrosis of the lung, liver, and kidney, suggesting a functional role for cFN containing EDA in these biological processes.

Constitutive Expression of EDA+-FN

To determine the functional role of EDA+-FN in atherosclerosis, we generated several novel mutant strains of atherosclerotic apolipoprotein E–deficient (Apoe–/–) mice: (1) EDAfl/flApoe–/– mice, which constitutively express EDA+-FN; (2) EDA−/−Apoe–/– mice, which completely lack EDA+-FN; (3) EDAfl/flTLR4−/−Apoe–/– mice, which constitutively express EDA+-FN but lack TLR4; (4) EDA−/−TLR4−/−Apoe–/– mice, which lack EDA+-FN and TLR4; (5) TLR4−/−Apoe–/– mice, which lack TLR4; and (6) control Apoe–/– mice, which express low levels of EDA+-FN, as well as other FN splicing variants.

Herein, we provide evidence for the first time that TLR4 contributes to EDA+-FN–mediated atherosclerosis exacerbation in Apoe–/– mice. Furthermore, we show that EDA+-FN colocalizes with TLR4 on macrophages in murine aortic lesions and human coronary atherosclerotic plaques.

Materials and Methods

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

Results

Constitutive Expression of EDA+-FN Promotes Progression of Atherosclerosis, Whereas Deletion of EDA Protects Against Atherosclerosis, in Apoe–/– Mice

To determine the functional role of EDA+-FN in atherosclerosis, we generated several novel mutant strains of Apoe–/– mice as described in Materials and Methods. Both male and female EDA−/−Apoe–/– mice (which lack EDA+-FN), EDAfl/flApoe–/– mice (which constitutively express EDA+-FN), and control Apoe–/– mice were first fed a chow diet for 6 weeks and then a high-fat Western diet for 14 weeks. Using ELISA, we first measured plasma levels of EDA+-FN. As expected at baseline, EDA+-FN signaling.

Table. Plasma Total Cholesterol and Triglyceride Levels in Male and Female Mice Fed a High-Fat Western Diet for 14 Weeks

<table>
<thead>
<tr>
<th>Strains</th>
<th>Total Cholesterol Levels, mg/dL</th>
<th>P Value vs Apoe−/−</th>
<th>Triglyceride Levels, mg/dL</th>
<th>P Value vs Apoe−/−</th>
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</thead>
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<tr>
<td>Male</td>
<td></td>
<td></td>
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<tr>
<td>Apoe−/−</td>
<td>597±54</td>
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<td>186±11</td>
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<td>EDA−/−Apoe−/−</td>
<td>645±57</td>
<td>P=0.8</td>
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<tr>
<td>TLR4−/−Apoe−/−</td>
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<td>P=0.6</td>
<td>183±16</td>
<td>P=0.8</td>
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<tr>
<td>EDA−/−TLR4−/−Apoe−/−</td>
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<td>P=0.4</td>
<td>196±19</td>
<td>P=0.7</td>
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<tr>
<td>EDAfl/flTLR4−/−Apoe−/−</td>
<td>604±60</td>
<td>P=0.8</td>
<td>177±24</td>
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<td>Female</td>
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<tr>
<td>Apoe−/−</td>
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<tr>
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<tr>
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<td>110±16</td>
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<td>P=0.9</td>
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<td>P=0.3</td>
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<tr>
<td>EDAfl/flTLR4−/−Apoe−/−</td>
<td>402±51</td>
<td>P=0.2</td>
<td>117±22</td>
<td>P=0.3</td>
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</tbody>
</table>

Values are represented as means±SEM. N=8 to 10/group. Apoe−/− indicates apolipoprotein E–deficient mouse; EDA, extra domain A; and TLR4, Toll-like receptor 4.
was absent in the plasma of 

$EDA^{-/-}Apoe^{-/-}$ mice and elevated by 3-fold in $EDA^{+/+}Apoe^{+/-}$ mice compared with $Apoe^{+/-}$ mice ($P<0.05$; Table I in the online-only Data Supplement). Plasma levels of $EDA^{-/-}FN$ increased progressively after 14 weeks of high-fat Western diet in both $Apoe^{-/-}$ and $EDA^{+/+}Apoe^{+/-}$ mice but remained significantly higher in $EDA^{+/+}Apoe^{+/-}$ mice compared with $Apoe^{+/-}$ mice ($P<0.05$, Table I in the online-only Data Supplement). Irrespective of sex, plasma cholesterol and triglyceride levels were similar in the $EDA^{-/-}Apoe^{-/-}$, $EDA^{+/+}$ $Apoe^{+/-}$, and $Apoe^{+/-}$ mice fed a high-fat diet for 14 weeks (Table), suggesting that inclusion or deletion of EDA in FN does not affect plasma lipid levels. Next, we compared the extent of atherosclerosis in whole aortae by staining with Oil Red O and quantifying en face lesion area. Both male and female $EDA^{+/+}Apoe^{+/-}$ mice exhibited significantly larger lesion areas, whereas male and female $EDA^{-/-}Apoe^{-/-}$ exhibited significantly reduced lesion areas compared with male and female $Apoe^{-/-}$ mice ($P<0.05$, $N=10–12$ mice/group; Figure 1A and 1B).

Next, we measured the interstitial collagen content. Picrosirius red staining of lesions in the aortic sinus revealed similar collagen content (Figure I in the online-only Data Supplement) among genotypes. Additionally, plasma tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels were comparable among genotypes (Figure II in the online-only Data Supplement).

Constitutive expression of $EDA^{+/+}FN$ increases macrophage infiltration within atherosclerotic plaques of $Apoe^{-/-}$ mice, whereas deletion of EDA reduces plaque inflammation. To determine whether $EDA^{+/+}FN$ enhances inflammatory cell recruitment to atherosclerotic plaques, we...
quantified macrophage infiltration (Mac3-positive area) within plaques of the aortic sinus by immunohistochemistry in mice fed a high-fat diet for 14 weeks. EDAfl/flApoe−/− mice exhibited significant increase in absolute Mac3-positive area, as well as % of total lesion area covered by Mac3-positive cells, whereas EDA−/−Apoe−/− mice had significantly reduced Mac3-positive area when compared with Apoe−/− mice (P<0.05; Figure 2A). Total leukocyte counts were similar among genotypes (Table II in the online-only Data Supplement).

Exogenous EDA+-FN Promotes Uptake of Native Low-Density Lipoprotein Complexes Containing Heparin and Collagen

To determine whether there was a difference in uptake of modified low-density lipoprotein (LDL) by macrophages, we incubated bone marrow–derived macrophages from EDAfl/flApoe−/−, EDA−/−Apoe−/−, and Apoe−/− mice with acetylated LDL and then stained with Oil Red O. We found that foam cell formation and acetylated LDL uptake was comparable among EDAfl/flApoe−/−, EDA−/−Apoe−/−, and Apoe−/− mice after 24 hours (Figure 2B). Additionally, foam cell formation and acetylated LDL uptake was comparable in EDA−/−Apoe−/− macrophages treated either in presence or absence of exogenous human cFN, which contains EDA (EDA−/FN; Figure III in the online-only Data Supplement). Previous in vitro studies have suggested that FN interaction with other ECM proteins, such as collagen and glycosaminoglycans, may enhance uptake of LDL.24,25 Therefore, we determined whether LDL complexes (native LDL–collagen–heparin) accumulate more in the macrophages in the presence of cFN. Interestingly, we found a significant increase in foam cell formation and LDL complex uptake in EDA−/−Apoe−/− macrophages treated with LDL–heparin–collagen–cFN complexes compared with LDL–heparin–collagen complexes (Figure 2C). Foam cell formation and LDL uptake was comparable in EDA−/−Apoe−/− macrophages treated with LDL or LDL–cFN (Figure 2C).

Figure 2. Cellular fibronectin containing extra domain A (EDA–FN) enhances macrophage cells infiltration but not foam cell formation. A, Top, Representative photomicrographs stained for macrophages (Mac3-positive cells stained as brown) and counterstained with hematoxylin (blue). Scale bar=200 μm. Bottom, Quantification. N=8 mice/group. Each dot represents a single mouse. Value for each mouse represents a mean of 16 fields from 4 serial sections (each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm). B, Left, Staining of purified bone marrow–derived macrophages from female EDA−/−Apoe−/−, EDAfl/flApoe−/−, and control Apoe−/− mice with Oil Red O 24 hour after incubating them with acetylated low-density lipoprotein (LDL; 100 μg/mL). Right, Quantification of total cholesterol. C, Left, Staining of purified bone marrow–derived macrophages from female EDA−/−Apoe−/− mice with Oil Red O 24 hour after incubating them with LDL (50 μg/mL) under different conditions. Right, Quantification of total cholesterol. Values are mean±SEM. N=5 to 6 mice/group. Apoe−/− indicates apolipoprotein E-deficient mouse; cFN, cellular fibronectin; and NS, non significant.
EDA⁺-FN Promotes Progression of Atherosclerosis Via TLR4

Because EDA⁺-FN interacts with TLR4 in vitro,²⁰ we tested the hypothesis that EDA⁺-FN promotes progression of atherosclerosis through the TLR4 signaling pathway. We used a genetic approach and generated Apoe⁻⁻, EDAfl/flApoe⁻⁻, and EDA⁻⁻Apoe⁻⁻ mice on a TLR4-deficient background. Controls included Apoe⁺⁺, EDAfl/flApoe⁻⁻, and EDA⁻⁻Apoe⁻⁻ littermates, respectively. Female mice were fed a high-fat Western diet for 14 weeks. Deletion of TLR4 in Apoe⁻⁻, EDAfl/flApoe⁻⁻, and EDA⁻⁻Apoe⁻⁻ mice did not alter plasma cholesterol or triglyceride levels when compared with controls (Table). Deletion of TLR4 in EDAfl/flApoe⁻⁻ mice significantly reduced total lesion area in the aorta, as well as cross-sectional lesion area in the aortic sinus, compared with EDAfl/flApoe⁻⁻ mice (P<0.05; Figure 3A and 3B). Interestingly, no significant differences in plaque area in the aorta or aortic sinus were observed between EDA⁻⁻TLR4⁻⁻Apoe⁻⁻ mice and control

Figure 3. Toll-like receptor 4 (TLR4) contributes to cellular fibronectin containing extra domain A (EDA⁺-FN)–mediated accelerated atherosclerosis in the aorta and aortic sinus of female mice fed a high-fat Western diet for 14 weeks. A, Left, Oil Red O staining of the representative aortae. Right, Quantification of en face lesion (N=8–12 mice/group). B, Left, Representative photomicrographs of VerHoeffs/Van Geison-stained aortic sinuses. Scale bar=200 μm. Right, Quantification of lesion in the cross section area of aortic sinuses (N=8–10 mice/group). C, Top, Representative photomicrographs stained for macrophages (Mac3-positive cells stained as brown). All the sections were counterstained with hematoxylin (blue). Scale bar=200 μm. Bottom, Quantification of Mac3-positive area. N=8 mice/group. Each dot represents a single mouse.
EDA−/−Apoe−/− mice (Figure 3A and 3B). Together these results suggest that EDA−/−FN requires TLR4 for atherosclerosis exacerbation. Next, using serial sections, we measured macrophage (Mac3-positive cells) infiltration within plaques of the aortic sinus by immunohistochemistry. Significant decrease in absolute Mac3-positive area and % of total lesion area covered by Mac3-positive cells were observed in EDA−/−TLR4−/−Apoe−/− mice compared with control EDA+/+TLR4+/+Apoe+/+ mice (P<0.05, Figure 3C). Mac3-positive area was significantly decreased in TLR4−/−Apoe−/− mice compared with Apoe−/− mice (P<0.05; Figure 3C). No significant differences in Mac3-positive area were observed between EDA+/−TLR4+/−Apoe−/− and EDA+/−Apoe−/− mice (Figure 3C).

EDA−/−FN Promotes NFkB p65–Mediated Inflammation in Bone Marrow–Derived Macrophages

We next determined whether the interactions of exogenous EDA−/−FN and TLR4 upregulate canonical NFκB p65 signaling. Bone marrow–derived macrophages from EDA−/−Apoe−/− and EDA+/−TLR4+/−Apoe−/− mice were stimulated for 24 hours in the presence of human cFN (cFN; 0–50 μg/mL), which contains the EDA. We then measured levels of phospho-NFκB p65 and NFκB p65 in cell lysates and the inflammatory cytokines TNF-α and IL-1β in medium. At doses of 5, 10, and 50 μg/mL of cFN, immunoblotting experiments revealed a significant linear increase in phospho-NFκB p65/total NFκB p65, TNF-α and IL-1β protein levels in cFN–treated bone marrow–derived macrophages from EDA+/−Apoe−/− mice compared with untreated control EDA+/−Apoe−/− mice (Figure IV in the online-only Data Supplement). Phospho-NFκB p65/total NFκB p65, TNF-α, and IL-1β protein levels were comparable in phorbol myristate acetate–treated EDA+/−TLR4+/−Apoe−/− and EDA−/−Apoe−/− macrophages, which strongly suggests that the observed in vitro effects were not simply mediated by TLR4 deletion, but rather by a specific effect of EDA−/−FN (Figure IV in the online-only Data Supplement).

EDA−/−FN Colocalizes With TLR4 on Macrophages in Human and Mice Atherosclerotic Plaques

To determine whether EDA−/−FN colocalizes with TLR4 on macrophages within human atherosclerotic plaques, we performed triple-labeling fluorescent immunostaining.
of autopsy samples from patients with coronary artery disease. In agreement with previous reports,\textsuperscript{6,18} EDA\textsuperscript{+}-FN was abundantly expressed within human coronary artery atherosclerotic lesions (Figure 5A–5F). Immunostaining revealed marked infiltration of Mac3- and TLR4-positive cells within the lesions, whereas staining was virtually absent in controls ($P<0.0001$, N=4/group, Pearson’s correlation; Figure 5). Double-labeling immunostaining showed that EDA\textsuperscript{+}-FN colocalizes with both TLR4 (Figure 5D) and macrophages (Figure 5F). Triple-labeling immunofluorescence staining for EDA\textsuperscript{+}-FN, macrophages, and TLR4 suggested that EDA\textsuperscript{+}-FN colocalizes with TLR4 expressed on macrophages (Figure 5A–5C). Similarly, we found colocalization of EDA\textsuperscript{+}-FN with TLR4 on macrophages within aortic lesions of Apoe\textsuperscript{−/−} mice fed a high-fat Western diet for 14 weeks (Figure 6).

Discussion

Although hypercholesterolemia remains a major risk factor for progression of atherosclerosis and a major therapeutic target, chronic inflammation is a critical contributor to the development of atherosclerosis. Moreover, it is widely accepted that both adaptive and innate immune receptors, such as TLR4, are known to mediate progression of atherosclerosis by promoting inflammation.\textsuperscript{23,26,27} We, herein, provide evidence for the first time that EDA\textsuperscript{+}-FN, a variant of FN that contains the alternatively spliced EDA domain, colocalizes with TLR4 and macrophages within human coronary artery plaques and aortic lesions in atheroprone Apoe\textsuperscript{−/−} mice. Furthermore, using several novel mutant strains of Apoe\textsuperscript{−/−} mice, we showed that EDA\textsuperscript{+}-FN promotes atherosclerosis exacerbation partially through TLR4.

Despite previous evidence suggesting that EDA\textsuperscript{+}-FN is proatherogenic,\textsuperscript{18,19} the precise mechanism by which
EDA+-FN promotes atherosclerosis was unclear. Tan et al proposed that reduced atherosclerosis in EDA-/-Apoe-/- mice fed a high-fat Western diet for 8, 12, and 16 weeks may be because of decreased total plasma cholesterol, but the differences in cholesterol levels reported were modest. In another study in which atherosclerosis was induced by an atherogenic diet containing sodium cholate, Babaev et al reported reduced atherosclerosis in both EDA-/- mice and EDA-/- mice compared with wild-type (C57BL/6J) mice. However, no significant differences in total cholesterol or triglyceride levels were observed between EDA-/- mice and EDA-/- mice fed the atherogenic diet for 8, 14, or 18 weeks. Because genetically induced atherosclerosis mouse models, such as the Apoe-/- mouse, are considered to be more clinically relevant and reproducible, we generated EDA-/- mice and EDA-/- mice on Apoe-/- genetic background. Irrespective of the sex, we found significantly reduced atherosclerosis in the aorta and aortic sinus of EDA-/-Apoe-/- mice (which lack EDA-/-FN) and exacerbated atherosclerosis in EDA-/-Apoe-/- mice (which constitutively express EDA-/-FN) when compared with control Apoe-/- mice. However, plasma cholesterol and triglyceride levels were similar among EDA-/-Apoe-/-, EDA-/-Apoe-/-, and Apoe-/- mice. Together, these results suggest that atherosclerosis exacerbation in EDA-/-Apoe-/- mice was not because of altered plasma cholesterol and triglyceride levels but rather because of an alternative mechanism. Indeed, several murine studies have shown that increased atherosclerotic lesions do not always correlate with plasma cholesterol and LDL levels.

Chronic inflammation triggered by the innate immune system is recognized as a key driving force for progression of atherosclerosis. Our studies show that EDA+-FN promotes macrophage infiltration within athrosclerotic plaques of the aortic sinus. Intracellular and extracellular compartments contribute to lipid deposition within atherosclerotic lesions. In animal models and in humans, it is known that, within atherosclerotic lesions, monocyte-derived macrophages can take up modified extracellular lipids, thereby resulting in increased lipid deposition in the vessel wall. In our studies with bone marrow–derived macrophages, we found that uptake of modified lipoproteins was comparable in EDA-/-Apoe-/-, EDA-/-Apoe-/-, and Apoe-/- mice. However, the in vivo microenvironment within the lesions in which macrophages reside is completely different compared with isolated cells. We hypothesize that the presence of EDA+-FN in the arterial wall in combination with collagen and glycosaminoglycans may enhance uptake of LDL by macrophages within the lesions. Indeed, we found that exogenous cFN enhanced macrophage uptake of LDL–heparin–collagen complexes. Our findings are in agreement with previous observations that LDL when incubated with heparin, FN, and collagen is avidly taken by
Because the protein components that were used to make LDL complexes are present in the vascular wall, we suggest that EDA-FN may potentiate foam cell formation during atherogenesis. We speculate that this could be one of the mechanisms by which EDA-fl/fl Apoe−/− mice are proatherogenic, independent of alterations in plasma cholesterol or lipid levels.

Herein, we also showed for the first time the role of TLR4 in EDA-FN-mediated atherosclerosis exacerbation. Previously, it was demonstrated that the EDA, but not other domains, of FN activates human TLR4 expressed in HEK293 (human embryonic kidney) cells, which normally lack TLR4.20 Like lipopolysaccharide, EDA activation of TLR4 requires MD-2 (myeloid differentiation factor-2), an accessory protein associated with extracellular domain A of TLR4 and required for TLR4-dependent lipopolysaccharide response.20 We now provide in vivo evidence that EDA-FN promotes progression of atherosclerosis through a mechanism that is partially dependent on TLR4. Multiple endogenous ligands (eg, heat-shock proteins, fibrinogen, and fibrin) have been shown to activate TLR4 and generate an inflammatory response. We found that TLR4 deficiency in EDA-fl/fl Apoe−/− or Apoe−/− mice significantly reduced lesion progression, concomitant with decreased infiltration of macrophages within the aortic sinus, although plasma cholesterol and triglyceride levels were comparable. Our results are consistent with findings from other groups who have shown that the atheroprotective effects of MyD88 or TLR4 deficiency are not because of altered serum cholesterol or lipoproteins but rather to reduced inflammation.23 Of note, TLR4 deficiency did not significantly alter lesion size in EDA−/− Apoe−/− mice suggest that EDA-FN could be one of the major endogenous ligands that promotes atherogenesis through the TLR4 signaling pathway.

TLR4 is expressed on multiple cell types that might contribute to the proatherogenic role of EDA-FN. Herein, using bone marrow–derived macrophages, we demonstrate that exogenous cFN potentiated the phosphorylation of NFKB p65, which is a component of the canonical signaling pathway downstream of TLR4 and has been reported to promote atherosclerosis.32,33 Our in vitro studies support a mechanistic model in which lesion macrophages, through TLR4, interact with EDA-FN in the ECM and, thereby, promote inflammatory response that may then amplify the inflammatory microenvironment within atherosclerotic lesions by promoting additional monocyte entry. Although our in vitro mechanistic studies suggest a role for TLR4 on macrophages that may contribute to EDA-FN–mediated inflammation and, thereby, atherosclerosis exacerbation, additional murine studies using either endothelial- or bone marrow–specific deletion of TLR4 will be required to define the specific cell types responsible for the TLR4-dependent effects of EDA-FN on atherosclerosis in vivo. Finally, we provide evidence for the first time that EDA-FN colocalizes with TLR4 on macrophages within human coronary atherosclerotic plaques and in murine aortic lesions. Although our studies indicate that TLR4 signaling significantly contributes to EDA-FN–mediated inflammation during atherosclerosis, it remains possible that some of the proinflammatory effects of EDA-FN are TLR4-independent, perhaps mediated by binding sites for leukocyte integrins α4β1 and α9β1 in the EDA domain.33 Additional studies will be required to determine whether disruption of EDA-FN–integrin interactions in vivo prevents monocyte recruitment and subsequent atherosclerotic lesion progression.

In summary, our studies unequivocally demonstrate that EDA-FN is proatherogenic in mouse models of atherosclerosis. Importantly, we provide genetic evidence for the first time that EDA-FN/TLR4 signaling enhances recruitment of monocytes/macrophages into developing plaques, thereby promoting progression of atherosclerosis. The abundant expression of EDA-FN in human atherosclerotic plaques and the mechanistic insights provided by the current study may open new arenas for the prevention and treatment of atherosclerosis in patients at high risk for coronary heart disease.

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Disclosures
None.

References
Cellular fibronectin containing extra domain A (EDA\textsuperscript{+}-FN) isoforms are abundant in the extracellular matrix of atherosclerotic arteries but absent from healthy arteries. We show that exogenous cellular fibronectin stimulates macrophage uptake of low-density lipoprotein–heparin–collagen complexes, suggesting that extracellular matrix rich in EDA\textsuperscript{+}-FN may play a role in cellular lipid accumulation in atherosclerotic lesions. Additionally, we demonstrate for the first time that EDA\textsuperscript{+}-FN colocalizes with Toll-like receptor 4 on macrophages in human coronary artery atherosclerotic plaques, suggesting a proinflammatory role for EDA\textsuperscript{+}-FN in atherosclerosis exacerbation. The abundant presence of EDA\textsuperscript{+}-FN in human atherosclerosis and the mechanistic insights provided by the current study raises possibility to target EDA\textsuperscript{+}-FN that may show benefit in patients at high risk of atherosclerosis.
Fibronectin Splicing Variants Containing Extra Domain A Promote Atherosclerosis in Mice Through Toll-Like Receptor 4
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Fibronectin splicing variants containing extra domain A promote atherosclerosis in mice through Toll-like receptor 4

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Animals

EDA<sup>fl/fl</sup> mice are a mutant strain in which the wild-type EDA exon in the FN gene was replaced with a “floxed (loxP-flanked allele)” EDA exon having both the 5′ and 3′ optimized splice sites at splicing junctions to prevent alternative splicing as described.1 As a result, the EDA exon is included in the FN mRNA, leading to constitutive expression of EDA<sup>+</sup>-FN in all cells in which FN is normally expressed, including liver (the major source of plasma FN), endothelial cells, and macrophages.1,2 EDA<sup>fl/fl</sup> mice were crossed with a CRE-recombinase transgenic mouse (C57BL/6J background) to obtain EDA<sup>-/-</sup> progeny that were intercrossed to obtain EDA<sup>+/-</sup> mice.1 To generate EDA<sup>+/-</sup>:Apoe<sup>+/-</sup> mice, EDA<sup>+</sup> mice1 (backcrossed >15 times to C57BL6J) were crossed to Apoe<sup>+</sup> mice (The Jackson Laboratory, Bar Harbor, ME). EDA<sup>fl/fl</sup> mice1 (backcrossed >15 times to C57BL/6J) were crossed to Apoe<sup>+</sup> mice (The Jackson Laboratory, Bar Harbor, ME) to generate EDA<sup>fl/fl</sup>:Apoe<sup>+</sup> mice. To generate EDA<sup>fl/fl</sup>TLR4<sup>+</sup>Apoe<sup>+</sup> mice, EDA<sup>fl/fl</sup>Apoe<sup>+</sup> mice were crossed to TLR4<sup>+</sup>Apoe<sup>+</sup> mice. Similarly, to generate EDA<sup>+</sup>TLR4<sup>+</sup>Apoe<sup>+</sup> mice, EDA<sup>+</sup>/Apoe<sup>+</sup> mice were crossed to TLR4<sup>+</sup>Apoe<sup>+</sup> mice. Whenever possible, littermate control mice were studied; however, due to allelic complexity some groups included animals from multiple congenic and age-matched litters. Mice were genotyped by PCR according to protocols from the Jackson laboratory and as described previously.1 All the mice used in the present study are on C57BL/6J background. The University of Iowa Animal Care and Use Committee approved all procedures.

Animal diet feeding and preparation of tissues

Both male and female mice (EDA<sup>fl/fl</sup>Apoe<sup>+</sup>, EDA<sup>+</sup>Apoe<sup>+</sup>, Apoe<sup>+</sup>, EDA<sup>fl/fl</sup>TLR4<sup>+</sup>Apoe<sup>+</sup>, EDA<sup>+</sup>TLR4<sup>+</sup>Apoe<sup>+</sup>, and TLR4<sup>+</sup>Apoe<sup>+</sup>) were fed a high-fat Western diet containing 20% milk fat and 0.2% cholesterol (Harlan Teklad) beginning at 6 weeks of age until they were sacrificed at 5 months of age (i.e., 14 weeks on high-fat Western diet). Blood samples were collected in heparinized tubes by retro-orbital plexus puncture after overnight fasting. Before sacrificing, mice were anesthetized with 100 mg/Kg ketamine/10 mg/Kg xylazine and perfused via the left ventricle with 10 ml PBS followed by 10 ml of 4% paraformaldehyde under physiological pressure. After perfusion, aorta was isolated, dehydrated for 5 min in 70% alcohol and stained with Oil Red O. Hearts containing aortic roots were carefully dissected and fixed overnight in 4% paraformaldehyde prior to embedding in paraffin.
Extent and composition of atherosclerotic lesions
To measure the extent of atherosclerosis, whole aortae were isolated and stained with Oil Red O and en face lesion area was measured by morphometry using NIH ImageJ software. To quantify lesions in the aortic sinus, serial cross-sections of 5 µm were cut through the aorta beginning at the origin of the aortic valve leaflets and stained by VerHoeffs/Van Gieson method. Cross-sectional lesion area from each mouse was calculated using the mean value of 4 sections (each 80 µm apart, beginning at the aortic valve leaflets and spanning 320 µm) as described previously. NIH ImageJ software was used for quantification.

Picrosirius red staining for collagen type III and I
To quantify interstitial collagen within the lesions of the aortic sinus, serial cross-sections of 5 µm were stained with Picrosirius red method. Briefly, serial formalin-fixed sections were stained with Weigert’s haematoxylin. Sections were then washed in running tap water for 10 minutes followed by incubation for 4 hours in a freshly prepared 0.1% solution of Sirius Red F3B (Sigma-Aldrich, USA) in saturated aqueous picric acid. After rinsing twice in 0.01 N HCl and distilled water, sections were dehydrated and mounted in Permount (Vector Laboratories). Picrosirius red staining was analyzed by polarization microscopy. NIH ImageJ software with a defined threshold (minimum 100 and maximum 200) was used for quantification. A mean for each mouse was calculated from using the mean value of 4 sections (each 80 µm apart, beginning at the aortic valve leaflets and spanning 320 µm).

Mac3 staining of murine samples
Tissue preparation and histochemical staining were performed as described. Antigen retrieval was performed prior to immunohistochemical staining. Briefly, slides were incubated with blocking reagent followed by rat anti-mouse mac-3 for macrophages [BD Pharmingen] in the presence of 5% rabbit serum overnight at 4°C. The following day, slides were rinsed and stained by biotin-conjugated rabbit anti-rat Ig, avidin-linked enzyme peroxidase complex, and 3, 3′-diaminobenzidine as substrate. Slides were counterstained with hematoxylin, dehydrated, and examined under a light microscope (Zeiss). Incubation without primary antibodies and/or with isotype-matched immunoglobulins was used as a negative control for immunostaining. NIH ImageJ was used for lesion quantification and expressed as the area of positive immunostaining. A mean for each mouse was calculated using the mean value of 4 sections (each 80 µm apart, beginning at the aortic valve leaflets and spanning 320 µm).

Quantification of cellular EDA⁺-FN in plasma samples
Cellular EDA⁺-FN levels in the plasma were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, micro titer plates were coated overnight at 4°C with primary antibody for EDA⁺-FN (IST-9, 10 µg/mL, Abcam) diluted in 50 mM sodium carbonate buffer. 50 µl of plasma samples (diluted 1:2 in PBS) were incubated for 2 h in the coated wells at RT. After 5 washes biotinylated secondary antibody to FN (2 µg/ml diluted in blocking buffer) was added to wells and incubated for 1 h at RT. Following 5 washes avidin HRP solution (1:1000) in blocking buffer was added to wells and incubated for 30 minutes. Micro titer plates were washed 5 times, before adding 3, 3’, 5, 5’-tetramethylbenzidine substrate solution (Sigma) to the wells and the colorimetric reaction was stopped with H₂SO₄ (2M) after 10 min. Results were read in an ELISA microplate reader at A₄50 nm. Human cellular FN (Sigma) was used for standards.
**Determination of plasma total cholesterol and lipid levels**

Blood samples were collected in heparinized tubes by retro-orbital plexus puncture after overnight fasting. Plasma was separated by centrifugation and analyzed for total cholesterol (Infinity™ Cholesterol, Thermo scientific #TR13421) and triglyceride (Wako) levels by using enzymatic colorimetric assays according to the manufacturer’s instructions.

**Bone marrow-derived macrophage (BMDM) culture**

Femurs and tibias were removed aseptically from 10-15 week old female mice, bone marrow cavities were flushed, and BM cell suspensions seeded at 2 X10^6 cells/ml in DMEM (Dulbecco’s modified eagle medium) containing high glucose (25 mM), 4 mM glutamine, 1 MM pyruvate, 10% FCS, 1% penicillin, 1% streptomycin and 10ng/ml macrophage colony stimulating factors. After 7 days, macrophages were washed and recultured in the presence of 0-50 µg/mL cellular fibronectin (#F2518, Sigma) pre-coated tissue culture plates. Medium was collected for ELISA and stimulated macrophages were washed twice with PBS before protein extraction.

**Preparation of LDL complexes**

We prepared LDL complexes with cFN, denatured collagen and heparin according to protocols as described. Briefly, 100 µl of heparin (Sigma) was added to LDL (355 µg LDL suspended in 3 ml distilled water: Alfa Aesar, MA). To this mixture 500 µl of CaCl_2 (0.5 M) was added. The mixture became turbid after addition of CaCl_2. To this mixture either collagen (100 µg; Sigma) or collagen (100 µg) + cFN (100 µg; Sigma) was then added and volume adjusted to 3.8 ml and preparation left overnight at 4°C. Insoluble complexes were then centrifuged (1000g for 20 min) and pellets were resuspended in DMEM before addition to macrophage monolayers.

**Foam cell formation**

Acetylated LDL (100 µg/ml; Alfa Aesar, MA), acetylated LDL (100 µg/ml)+ cFN (10 µg/ml), LDL (50 µg/ml), LDL (50 µg/ml) + cFN (10 µg/ml), and LDL complexes were added to BMDMs for 24 hours. Cells were washed three times with PBS and fixed with 4 % formaldehyde. Lipids were stained using oil red O. Cells were cover slipped and foam cell images were obtained using Olympus BX51TF.

**Macrophage lipid parameters**

BMDM lipids were extracted with 2 ml of hexane/isopropyl alcohol (3:2, v/v) for 1 h at 4 °C. Lipid extracts were dried and redissolved in 100 µl of 1% Triton X-100 in chloroform. The samples were dried and resuspended in 100 µl of distilled water for 15 min at 37 °C in a water bath. For enzymatic measurements of total cholesterol (Infinity™ Cholesterol, Thermo scientific #TR13421) concentrations, 30 µl of samples were used. Protein was extracted from cells in 2ml of 0.3 M NaOH for 1hour at room temperature and quantified using a Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

**Protein extraction and immunoblotting**

BMDMs were lysed in ice-cold RIPA buffer. The lysates were spun down at 4°C (14,000rpm) for 15 minutes, and the supernatant was collected. Protein quantification was done by BCA method (Thermo scientific). Protein homogenates (40 µg) were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane. Membranes were blocked with 5% BSA plus 0.1% Tween 20 for 1 hour and incubated with following rabbit monoclonal antibodies NF-κB p65 (1:2000) (D14E12), Phospho-NF-κB p65 (Ser536) (93H1)(1:1000) from cell signaling technologies and polyclonal beta actin antibody (ab8227) (1:5000) from Abcam. Blots were incubated overnight at 4°C. The horseradish peroxidase-conjugated goat anti-rabbit (1:3000) antibodies were
visualized by super signal West Femto Maximum sensitivity substrate (Thermo scientific) on a rpi CLASSIC S-Ray film (248304). Densitometric analysis of the gels was done using ImageJ software.

**ELISA assay for TNF-α and IL-1β**

Supernatant medium from cFN treated and untreated macrophages were used for determination of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) with commercially available mouse ELISA kits (R&D Systems) according to the kit manufacturer’s instructions. Using same kits, plasma TNF-α and IL-1β levels were quantified.

**Triple labeling staining of human and mice samples**

**Human samples:** Human coronary artery tissue samples from autopsies of patients with a history of coronary artery disease were procured from the University of Iowa Decedent Center in accordance with guidelines established by the University of Iowa Institutional Review Board. The presence of atherosclerosis was confirmed by gross pathology. Samples were fixed in formalin, processed and paraffin-embedded for immunohistochemistry. Antigen retrieval was performed prior to immunohistochemical staining. Briefly, in the first step, 5-µm serial sections were blocked and incubated with mouse monoclonal antibody specific for EDA-FN (IST-9, 1:100, Abcam) and rabbit polyclonal antibody specific for TLR4 (H-80, 1:50, Santa Cruz) overnight at 4°C followed by secondary antibodies labeled with anti-mouse Alexa Fluor-568 and anti-rabbit Alexa Fluor-488 (1:400, Abcam) for 1hr at room temperature (RT). In the second step, sections were washed and blocked before incubating with mouse anti-human CD68 Alexa Fluor-647 antibody for macrophage (KP-1, 1:50, Santa Cruz) for 3hrs at RT. Slides were dehydrated, and examined using a Leica SP5 scanning laser confocal microscope at the core facility in the Department of Biology, University of Iowa. Confocal images (1,024 or 2,048 pixel arrays) were collected using a 10x/0.3 Fluotar, 20x/0.8 Plan Apo, or 63x/1.2 water immersion Plan Apo lens, with additional electronic zoom as needed, up to a resolution of 120nm xy pixel dimensions. To avoid cross-illumination of fluorophores in triple-labeled samples, images were captured sequentially using the indicated laser lines: Alexa Fluor-488 (Argon 488 nm); Alexa Fluor-568 (HeNe 543 nm); and Alexa Fluor-647 (HeNe 633 nm). Images were then false colored (Alexa Fluor-488, green; Alexa Fluor-568, red; Alexa Fluor-647, blue) and merged using Adobe Photoshop CS. **Murine samples:** Briefly, in the first step, 5-µm serial sections were blocked and incubated with rat anti-mouse monoclonal antibody specific for Mac-3 (CD107b, 1:50, BD Pharmingen) and rabbit anti-mouse polyclonal antibody specific for TLR4 (H-80, 1:50, Santa Cruz) overnight at 4°C. The sections were washed and incubated with secondary antibodies labeled with anti-rat Alexa Fluor-647 and anti-rabbit Alexa Fluor-488 (1:400, Abcam) for 1hr at room temperature (RT). In the second step, sections were washed and blocked before incubating with mouse monoclonal antibody specific for EDA-FN (IST-9, 1:100, Abcam) for 3hrs at room temperature (RT) and after washing incubated with anti-mouse Alexa Fluor-568 for 1hr at room temperature (RT). Confocal images were taken as described above.

**Quantitative colocalization analysis**

Leica LAS AF software was used for quantitative analysis of fluorescence intensity and colocalization in confocal images of triple-labeled tissue sections. Three pair-wise comparisons (TLR4-EDA, TLR4-MAC, and EDA-MAC) were made for colocalization analysis in both control and experimental tissue sections. Pixel intensities in 25 × 25 µm regions-of-interest were plotted in 2D scatter plots (cytofluorograms) according to the gray scale values for the two channels being compared. To avoid contamination from autofluorescence and non-specific background staining, threshold and background levels were set to 40% for both channels. Pearson’s Correlation was used as a quantitative measure of fluorescence colocalization. A
value of +1 indicates a complete match of the displays in both color channels, and a value of -1 indicates no match. Measurements were obtained from four separate confocal optical sections per tissue. Statistical analysis comparing experimental and control tissues were performed using two-tailed T-tests.

Statistical analysis
Results are reported as mean or mean ± SEM. The number of experimental animals in each group was based on power calculations for the primary parameter with standard deviations taken from pilot data in mice. We used sample sizes of 10 to 12 mice per group with the following assumptions: \( \alpha = 0.05, \beta = 0.2 \) (power 80%), mean, standard deviation 20% of the mean. For statistical analysis, Prism Graph software package was used. Statistical comparisons were performed using one way analysis of variance followed by Bonferroni’s multiple comparison test. For measuring the effect of two factors simultaneously, two ways analysis of variance followed by Holm-Sidak multiple comparison test was used. \( P < 0.05 \) was considered statistically significant.

References
Online supplement

Fibronectin splicing variants containing extra domain A promote atherosclerosis in mice through Toll-like receptor 4
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Supplementary Table I. Plasma cellular EDA⁺-FN concentrations were measured from each mouse using sandwich ELISA. Value are expressed as mean ± SEM. N= 8-10 mice/group.

<table>
<thead>
<tr>
<th>Mice strains</th>
<th>Plasma cellular EDA⁺-FN (µg/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>6 weeks on chow diet</td>
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<tr>
<td>EDA⁺/Apoe⁻⁻</td>
<td>0</td>
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<tr>
<td>Apoe⁻⁻</td>
<td>1.5 ± 0.3*</td>
</tr>
<tr>
<td>EDA⁻⁻/Apoe⁻⁻</td>
<td>4.7 ± 0.6#</td>
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Supplementary Table II. Complete Blood Counts from 8- 9 weeks old female mice. Value are expressed as mean ± SEM. N= 8-10 mice/group. P= Non significant versus control Apoe⁻⁻ mice.

<table>
<thead>
<tr>
<th></th>
<th>EDA⁺⁻/Apoe⁻⁻</th>
<th>Apoe⁻⁻</th>
<th>EDA⁻⁻/Apoe⁻⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10³/µl)</td>
<td>11.0 ± 0.7</td>
<td>9.65 ± 0.41</td>
<td>10.9 ± 0.9</td>
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<tr>
<td>RBC (10⁶/µl)</td>
<td>8.8 ± 0.1</td>
<td>8.87 ± 0.18</td>
<td>9.1 ± 0.1</td>
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<tr>
<td>HGB (g/dL)</td>
<td>14.0 ± 0.2</td>
<td>14.0 ± 0.23</td>
<td>14.2 ± 0.1</td>
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<tr>
<td>HCT (%)</td>
<td>44.5 ± 0.4</td>
<td>44.4 ± 0.74</td>
<td>44.1 ± 0.3</td>
</tr>
<tr>
<td>Platelet (10³/µl)</td>
<td>1009 ± 75</td>
<td>1014 ± 83</td>
<td>936 ± 33</td>
</tr>
<tr>
<td>Neutrophil (10³/µl)</td>
<td>0.8 ± 0.04</td>
<td>0.96 ± 0.12</td>
<td>0.7 ± 0.08</td>
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<td>Lymphocytes (10³/µl)</td>
<td>9.6 ± 0.64</td>
<td>8.0 ± 0.37</td>
<td>9.6 ± 0.82</td>
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<tr>
<td>Monocytes (10³/µl)</td>
<td>0.3 ± 0.05</td>
<td>0.31 ± 0.06</td>
<td>0.3 ± 0.02</td>
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<tr>
<td>Eosinophils (10³/µl)</td>
<td>0.2 ± 0.04</td>
<td>0.39 ± 0.05</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>Basophils (10³/µl)</td>
<td>0.005 ± 0.003</td>
<td>0.005 ± 0.003</td>
<td>0.008 ± 0.003</td>
</tr>
</tbody>
</table>
Supplementary Figure I. Collagen staining in aortic sinus. A. Representative low magnification (4X) photomicrographs stained for collagen as visualized by polarization microscope. B. Representative high magnification (10X) photomicrographs of the boxed region (A) stained for collagen. C. Quantification of collagen positive area. Data is presented as mean ± SEM. N = 5-6 mice/group.
Supplementary Figure II. Plasma TNF-α and IL-1β levels. ELISA quantification of TNF-α and IL-1β in plasma from female EDA<sup>-/-</sup>Apoe<sup>-/-</sup>, EDA<sup>fl/fl</sup>Apoe<sup>-/-</sup>, and control Apoe<sup>-/-</sup> mice fed a high-fat Western diet for 14 weeks. Data is presented as mean ± SEM. N = 10/group.
Supplementary Figure III. Left panel shows staining of purified bone marrow-derived macrophages from female EDA<sup>-/-</sup>-Apoe<sup>-/-</sup> mice with Oil Red O 24 hour after incubating them with acLDL with or without exogenous cFN. Right panel shows quantification of total cholesterol. Values are mean ± SEM. N=6 mice/group. NS= non significant.
Supplementary Figure IV. Cellular FN promotes inflammation in macrophages through TLR4. Bone marrow-derived macrophages from EDA<sup>−/−</sup>Apoe<sup>−/−</sup> and EDA<sup>−/−</sup>TLR4<sup>−/−</sup>Apoe<sup>−/−</sup> were stimulated with 20 ng/mL of phorbol myristate acetate in presence or absence of cFN (10µg/mL) for 24 hours. A. Representative immunoblots in top panel shows expression of phosphorylated- NFκB p65, total NFκB p65 and β-actin. Bar diagram in middle and bottom panels represent quantification. β-actin was used as loading control. N = 4 mice/group. B&C. ELISA quantification of TNF-α and IL-1β in supernatant medium from cFN treated and untreated macrophages. Data is presented as mean ± SEM. N = 4 mice/group.