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TRAF3IP2 mediates atherosclerotic plaque development and vulnerability in *ApoE*$^{-/-}$ mice

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Abstract

Background and aims—Atherosclerosis is a major cause of heart attack and stroke. Inflammation plays a critical role in the development of atherosclerosis. Since the cytoplasmic adaptor molecule TRAF3IP2 (TRAF3-Interacting Protein 2) plays a causal role in various autoimmune and inflammatory diseases, we hypothesized that TRAF3IP2 mediates atherosclerotic plaque development.

Methods—*TRAF3IP2/ApoE* double knockout (*DKO*) mice were generated by crossing *TRAF3IP2*$^{-/-}$ and *ApoE*$^{-/-}$ mice. *ApoE*$^{-/-}$ mice served as controls. Both *DKO* and control mice were fed a high-fat diet for 12 weeks. Plasma lipids were measured by ELISA, atherosclerosis by *en face* analysis of aorta and plaque cross-section measurements at the aortic valve region, plaque necrotic core area, collagen and smooth muscle cell content by histomorphometry, and aortic gene expression by RT-qPCR.

Results—The plasma lipoprotein profile was not altered by *TRAF3IP2* gene deletion in *ApoE*$^{-/-}$ mice. While total aortic plaque area was decreased in *DKO* female, but not male mice, the plaque necrotic area was significantly decreased in *DKO* mice of both genders. Plaque collagen and smooth muscle cell contents were increased significantly in both female and male *DKO* mice compared to respective controls. Aortic expression of proinflammatory cytokine (Tumor necrosis

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Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

factor α, TNFα), chemokine (Chemokine (C-X-C motif) Ligand 1, CXCL1) and adhesion molecule (Vascular cell adhesion molecule 1, VCAM1; and Intercellular adhesion molecule 1, ICAM1) gene expression were decreased in both male and female *DKO* mice. In addition, the male *DKO* mice showed a markedly reduced expression of extracellular matrix (ECM)-related genes, including TIMP1 (Tissue inhibitor of metalloproteinase 1), RECK (Reversion-Inducing-Cysteine-Rich Protein with Kazal Motifs) and ADAM17 (A Disintegrin And Metalloproteinase 17).

Conclusions—TRAF3IP2 plays a causal role in atherosclerotic plaque development and vulnerability, possibly by inducing the expression of multiple proinflammatory mediators. TRAF3IP2 could be a potential therapeutic target in atherosclerotic vascular diseases.

Introduction

Atherosclerosis is an important contributing factor for cardiovascular diseases such as heart attack and stroke (1). It is one of the major leading causes of death in Western countries and its incidence is increasing alarmingly in developing countries (1). Atherosclerosis results in gradual narrowing of arterial lumen due to lipid accumulation and infiltration of immune cells into the subintimal space, and involves complex cellular and molecular interactions, leading to plaque development, rupture, and thrombosis (3). Although the major stages involved in atherosclerotic plaque development and stability have been well described, the molecular mechanisms underlying its pathogenesis are not fully understood. Identifying newer molecules and determining their role in atherosclerosis development and progression will help us better understand the disease process and the development of novel therapeutic strategies.

TRAF3-Interacting Protein 2 (TRAF3IP2) is a cytoplasmic adaptor molecule and activator of the transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1) in I kappa B kinase (IKK)- and c-Jun N-terminal kinase (JNK)-dependent manner (20, 21). NF-κB and AP-1 regulate the expression of various pro-atherogenic mediators, including proinflammatory cytokines, chemokines, adhesion molecules and ECM degrading MMPs, that play critical roles in atherosclerotic plaque formation and progression (30).

The major cell types involved in atherosclerotic plaque formation (*e.g.*, endothelial cells, smooth muscle cells, and macrophages) express TRAF3IP2 (39–44). High glucose and ox-LDL induce TRAF3IP2 expression and endothelial dysfunction *in vitro* (40, 44). Angiotensin II (AngII), aldosterone, interleukin-18 (IL-18), and advanced oxidation protein products (AOPPs) that play a role in atherogensis induce TRAF3IP2 expression *in vitro* (34, 39, 41–43), suggesting that TRAF3IP2 could play a role in atherosclerosis. Therefore, we hypothesized that TRAF3IP2 mediates atherosclerotic plaque development and progression.

Materials and methods

Generation of TRAF3IP2/ApoE double knockout (DKO) mice

All animal studies were approved by the Institutional Animal Care and Use Committee at Tulane University in New Orleans, LA, and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. *TRAF3IP2*$^{-/-}$ mice (C57Bl/6 background) were

previously described (8). *ApoE*$^{-/-}$ mice (C57Bl/6 background) were purchased from The Jackson Laboratory (Ann Harbor, ME). *TRAF3IP2* and *ApoE* heterozygous mice (*TRAF3IP2*$^{+/-}$/*ApoE*$^{+/-}$) were generated by crossing *TRAF3IP2*$^{-/-}$ and *ApoE*$^{-/-}$ mice, and the double knockout mice (*TRAF3IP2*$^{-/-}$/*ApoE*$^{-/-}$; *DKO*) by intercrossing *TRAF3IP2*$^{+/-}$/*ApoE*$^{+/-}$ mice. Lack of *TRAF3IP2* expression in aortas of *DKO* mice was confirmed by RT-qPCR (Table 2).

Genotyping

A genomic polymerase chain reaction (PCR) was used to identify the wild-type and mutant alleles of *TRAF3IP2* (WT product: 250bp; mutant product: 453bp) and *ApoE* (WT product: 145bp; mutant product: 250bp) (Supplemental Figure 1). *TRAF3IP2*: primer 1- 5'-CTGGCATGTTTTCTCTTGTTTC-3'; primer 2: 5'-GCCTTCTAAAGAAACTGGCTTC-3'; primer 3: 5'-CAATCATGTGTTCAGTCAGC-3'; primer 4: 5'-GCTCTATGGCTTCTGAGG-3'. The PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s. *ApoE*: primer 1: 5'-GCCTAGCCGAGGGAGAGCCG-3'; primer 2: 5'-TGTGACTTGGGAGCTCTGCAGC-3'; primer 3: 5'-GCCGCCCCGACTGCATCT-3'. PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 68°C for 40 s, and extension at 72°C for 60 s.

Diet

Eight week-old male and female *DKO* and *ApoE*$^{-/-}$ mice (n=10–15/groups) were fed a Western diet (42% of total calories from fat; 0.15% cholesterol; #TD88137, Harlan-Teklad diets) for twelve weeks. Body weights were recorded weekly and blood was collected by cardiac puncture prior to euthanasia.

Atherosclerosis quantification

Mice were anesthetized and perfused initially with saline and then with 4% paraformaldehyde plus 5% sucrose. Heart and entire aorta were dissected and fixed overnight in 4% paraformaldehyde plus 5% sucrose. Atherosclerosis was quantified by *en face* analysis of aorta and determination of plaque cross-sectional area at the aortic root. For *en face* analysis, adventitial fat was removed from the aorta and stained with Oil Red O and opened longitudinally, pinned *en face* and photographed. The total arterial surface area and total plaque were determined by Image-Pro PLUS v 6.0 software (Media Cybernetics Inc.). The extent of lesion development was determined as a percentage of the total area of the aorta that was occupied by Oil Red O-positive atherosclerotic lesions. For determination of plaque cross-sectional area, serial 6μm-thick sections from the entire aortic root area were stained with hematoxylin and eosin (H&E). Plaque cross-sectional area was determined by quantifying the plaque area in images (DP70 digital camera) using Image-Pro PLUS software. The mean value of plaque cross-sectional areas from 3 sections was used to estimate the extent of atherosclerosis in each animal.

Features of plaque stability

The necrotic core area was determined in aortic valve plaques by quantifying acellular area (H&E negative), which also contains cholesterol crystals. Aortic valve sections were stained

with Masson's trichrome, photographed and collagen positive area was quantified. Smooth muscle cell content in plaques was determined by immunohistochemistry of aortic valve sections. Sections were incubated with mouse alpha-smooth muscle actin (αSMA)-antibody (Millipore) or isotype-matched mouse IgG (Abcam), followed by secondary antibody and Avidin, Alexa Flour® 488 conjugate (ThermoFisher). Nuclei were stained with DAPI. Images were taken using a fluorescent microscope and αSMA-positive area in the plaques was quantified.

Quantitative real-time RT-qPCR

Mice were anesthetized and perfused with saline, the whole aorta was dissected and cleaned in RNA*later*® (ThermoFisher) and stored at −80°C until further use. Total RNA was isolated using Trizol reagent (Sigma). Total RNA (0.5 μg) was reverse transcribed into cDNA using a reverse transcription kit. Collagen, type I, α1 (ColIα1; Assay ID: Mm00801666), Collagen, type III, α1 (ColIIIα1; Assay ID: Mm1254476), Matrix metalloproteinase 2 (MMP2; Assay ID: Mm01253621), Matrix metalloproteinase 9 (MMP9; Assay ID: mm00600163), Matrix metalloproteinase (MMP14; Assay ID: Mm01318969), Tissue inhibitor of metalloproteinase 1 (TIMP1, Assay ID: Mm00441818), Reversion-Inducing-Cysteine-Rich Protein with Kazal Motifs (RECK, Assay ID: Mm01342144), A disintegrin and metalloproteinase domain 10 (ADAM10, Assay ID: Mm00545742), ADAM17 (Assay ID: Mm00456428), Tumor necrosis factor α (TNFα, Assay ID: Mm00443258), Interleukin-6 (IL-6, Assay ID: Mm00446191), IL-18 (Assay ID: Mm00434226), Intercellular adhesion molecule (ICAM1, Assay ID: Mm01175876), Vascular cell adhesion molecule 1 (VCAM1, Assay ID: Mm00449197), Monocyte chemoattractant protein 1 (MCP 1, Assay ID: Mm00441242), Chemokine (C-X-C) ligand 1 (CXCL1, Assay ID: Mm00433859), Angiotensin II receptor, type 1 (AGTR1A, Assay ID: Mm01957722) and Angiotensin II receptor, Type 2 (AGTR2; Assay ID: Mm01341373) mRNA levels were determined by RT-qPCR using TaqMan® probes (Applied Biosystems). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. 18S (Assay ID. Hs99999901) served as the endogenous invariant control, and all data were normalized to corresponding 18S levels.

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was determined by student's t-test. Differences are considered significant if the *p* value is less than or equal to 0.05.

Results

TRAF3IP2 gene deletion does not alter body weight gain and plasma lipid profile in ApoE$^{-/-}$ mice

Initial body weights were similar between *ApoE*$^{-/-}$ and *DKO* male mice, whereas *DKO* female mice had higher body weights compared to *ApoE*$^{-/-}$ female mice (20.67±0.49g *vs.* 18.2±0.28; $\boldsymbol{p}$ <0.01) (Supplemental Figure 2). Increases in body weight were similar between gender-matched *ApoE*$^{-/-}$ and *DKO* mice during the twelve-week Western diet feeding (5.82±0.39g *vs.* 5.46±0.65g with $p > 0.05$ for females; 10.20±1.15g *vs.* 10.70±0.76g with $p > 0.05$ for males) (Supplemental Figure 2). Plasma triglycerides, total cholesterol, VLDL/LDL cholesterol, and HDL cholesterol levels were similar in the gender-matched

ApoE$^{-/-}$ and *DKO* mice (Table 1), though *DKO* male mice showed a strong trend towards an increase in plasma HDL cholesterol levels (56.7±16.7 *vs.* 26.7±9.8; ***p* <0.10**) (Table 1).

TRAF3IP2 gene deletion reduced atherosclerosis in ApoE$^{-/-}$ female mice, but not in male mice

En face analysis of aorta revealed that *DKO* females had 42.8% reduced total plaque area when compared to *ApoE*$^{-/-}$ females after twelve weeks on a Western diet (5.2±2.8% vs 8.72±0.93%; $p < 0.05$; Figure 1Ai & iii), whereas no such changes were observed in the *DKO* male mice (Figure 1Aii & iv). Morphometric analysis of aortic root plaque revealed no significant change in plaque cross-sectional area between the *DKO* mice of both genders compared to their controls (Figure 1Bi–iv).

TRAF3IP2 gene deletion decreased plaque vulnerability in both ApoE$^{-/-}$ female and male mice, as evidenced by increased plaque stability features

H&E staining of aortic root plaques sections revealed 30% decrease in necrotic area in the Western diet-fed *DKO* female mice when compared with *ApoE*$^{-/-}$ female mice (33.66±2.86% *vs.* 48.08±4.96%; $p < 0.05$; Figure 2Ai). Similarly, *DKO* male mice showed 36% decrease in the necrotic core area in aortic root plaques when compared to that of *ApoE*$^{-/-}$ male mice (20.19±1.8% *vs.* 31.57±5.89%; $p < 0.05$; Figure 2Aii).

Masson's trichrome staining of aortic root plaques revealed a significant 49.9% increase in collagen content in *DKO* female mice when compared to that of *ApoE*$^{-/-}$ female mice (40.35±4.1% *vs.* 26.91±4.5%; $p < 0.05$; Figure 2Bi & iii), whereas a 148.6% increase was observed in *DKO* male mice when compared to that of *ApoE*$^{-/-}$ males (39.26±3.1% *vs.* 15.79±3.0%; $p < 0.01$; Figure 2B i & iii).

Immunohistochemistry for smooth muscle cell content in aortic root sections revealed a strong trend towards an increase in αSMA-positive SMC area in atherosclerotic lesions in *DKO* females compared to *ApoE*$^{-/-}$ female mice (28.14±6.4% *vs.* 13.89±2.12%; $p < 0.10$; Figure 2B ii & iv). The αSMA-positive area in atherosclerotic lesions was increased significantly by 419.5% in *DKO* males compared to that of *ApoE*$^{-/-}$ males (24.73±5.3% vs 4.76±0.65%; $p < 0.05$; Figure 2B ii & iv).

TRAF3IP2 gene deletion decreased aortic expression of proinflammatory cytokines, chemokines and adhesion molecules in ApoE$^{-/-}$ mice

CXCL1 mRNA levels were significantly decreased by 62% in aortas of *DKO* female mice compared to that of *ApoE*$^{-/-}$ female mice (Table 2), whereas a trend towards decrease was observed in TNFα, ICAM1, VCAM1 and AT2 gene expression (Table 2). ICAM1 and CXCL1 mRNA levels were decreased significantly by 85% and 80% respectively in aortas of Western diet-fed *DKO* male mice compared to that of *ApoE*$^{-/-}$ male mice, whereas a trend towards decrease was observed in IL-6, TNFα, VCAM1 and AT1A gene expression (Table 2).

TRAF3IP2 gene deletion altered aortic mRNA levels of ECM-related proteins in ApoE$^{-/-}$ mice

Gene expressions of TIMP1, RECK and ADAM17 were significantly decreased by 60%, 75%, and 76%, respectively, in aortas of *DKO* male mice compared to that of *ApoE*$^{-/-}$ male mice (Table 2). No significant changes were observed in gene expression of ECM-related proteins in aortas of *DKO* females, except for TIMP1 which showed a strong trend towards decrease, and MMP9 and ADAM10 showed a trend towards increase when compared to *ApoE*$^{-/-}$ female mice (Table 2).

Discussion

TRAF3IP2 was initially discovered as an upstream activator of NF-κB and AP-1 transcription factors (20, 21). It plays a critical role in IL-17 signaling, as it directly interacts with the IL-17 receptor to activate various downstream inflammatory pathways (7). TRAF3IP2 ubiquitinates the bound TNF receptor-associated factor 6 (TRAF6) and activates IKK, resulting in IκB phosphorylation, degradation and nuclear translocation of NF-κB (22). TRAF3IP2 also activates AP-1 via IKKγ/JNK interaction (39). Its activation increases the stability of mRNAs encoding chemokines like CXCL1 through TRAF2/TRAF5 complex (35). Our previous studies showed that inflammatory mediators such as IL-18, AngII, aldosterone, AOPPs, ox-LDL and ROS induce TRAF3IP2 expression, which in turn activates NF-κB and AP-1, and induction of proinflammatory cytokines in various cell types of the cardiovascular system (34, 39, 41–43). Previous studies from our laboratory have shown that silencing TRAF3IP2 by siRNA and shRNA decreased cardiac fibroblast migration and cardiomyocyte death *in vitro,* respectively (39, 41). Results from both animal and human studies showed elevated IL-18, IL-17, and AngII levels and activation of JNK and IKK signaling in atherosclerotic plaques (10–12, 23, 26, 28, 33, 37). Recent studies also showed that aldosterone promotes atherosclerosis and plaque inflammation (25). Elevated TRAF2, 5 and 6 protein levels were also reported in human atherosclerotic plaques (46). Together, these observations support a possible causal role for TRAFF3IP2 in atherogenesis.

Plaque stability is a critical determinant of its vulnerability to rupture. Stable plaques have lesser necrotic core with thicker fibrous caps, higher number of SMCs, and increased deposition of ECM proteins like collagens, whereas unstable plaques have larger necrotic area with thinner fibrous caps, and lower SMC and collagen content. In this study *TRAF3IP2* gene deletion increased features of plaque stability in both male and female *ApoE*$^{-/-}$ mice as evidenced by decreased necrotic core and increased SMC content and collagen levels. Size of necrotic core is influenced by the rate of monocyte recruitment, differentiation, foam cell formation and death (29). In a previous study, we showed that ox-LDL activates endothelial cell and monocyte attachment *in vitro* via TRAF3IP2-dependent increases in ICAM1 and VCAM1 expression (40). In the present study, aortic expression of ICAM1 and VCAM1 was reduced in the *DKO* mice, possibly suggesting that reduced endothelial cell activation in these mice might have resulted in reduced monocyte recruitment. These mice also showed significantly reduced CXCL1 expression, a chemokine known to mediate ox-LDL-mediated monocyte recruitment (45), further supporting our hypothesis. Matsuyama *et al.* showed that TRAF6 activation promotes monocyte

differentiation by regulating cell-ECM interactions suggesting that TRAF3IP2-null mice might have also had reduced monocyte differentiation due to reduced TRAF6 signaling (24).

The role of TRAF3IP2 in foam cell formation and apoptosis is not studied yet, but signaling cascades involving TRAF3IP2 were shown to regulate these critical steps. Activated NF-κB was reported in atherosclerotic plaques from human and animal models (6). Hematopoietic cell- and endothelial cell-specific inhibition of NF-κB resulted in decreased atherosclerosis, whereas macrophage- and myeloid-cell-specific inhibition of NF-κB resulted in increased atherosclerosis with elevated macrophage apoptosis (15). Studies showed that JNK pathway is critical for foam cell formation (32). The role of IL-17, an upstream activator of TRAF3IP2, in atherosclerosis is inconclusive, as the studies demonstrated contrasting effects (16). Though, cell-specific inhibition of signaling molecules regulated by TRAF3IP2 have shown divergent outcomes on plaque stability, alteration of these pathways by global knockdown of TRAF3IP2 might have altered signaling cascades towards increased plaque stability. Interestingly, the *DKO* mice showed reduced aortic expression of TNFα in both male and female mice, which could be one of the possible mechanisms involved in decreased necrotic core, as TNFα is an important mediator of macrophage apoptosis (38). In male mice, reduced aortic expression of IL-6, AT1A and ADAM17 along with TNFα might have further improved plaque stability features, as they were shown to play a casual role in atherosclerotic plaque development and vulnerability (14, 17, 36).

The progression of stable to unstable plaques is characterized by an increase in SMC apoptosis, resulting in reduced number of SMCs, which in turn reduces ECM deposition (2). Activated NF-κB was detected in SMCs from human atherosclerotic plaques (4), and both IKK/NF-κB and JNK/AP-1 play a role in VSMC apoptosis (13, 19, 27). JNK has also been shown to mediate TNFα-induced autophagy in human VSMCs (18). In this study, *TRAF3IP2* gene deletion increased plaque SMC and collagen content in both the genders but to a greater extent in males, thus providing the first evidence that TRAF3IP2 mediates vulnerable plaque formation. Perhaps, reduced JNK signaling due to *TRAF3IP2* gene deletion might have resulted in decreased SMC apoptosis and increased plaque stability features, nullifying the effects of reduced NF-κB signaling. TNFα is also an important mediator of SMC apoptosis (5) and decreased TNFα expression might have contributed to increased fibrous cap formation and a stable plaque in the *DKO* mice.

MMPs play a critical role in plaque stability, as they regulate ECM degradation and VSMC proliferation and migration. In the DKO mice of either gender, a trend towards increased MMP9 expression was observed along with reduced expression of its endogenous inhibitors, TIMP1 in female mice and RECK in male mice, suggesting a possible local increase in MMP activity in atherosclerotic plaques of the *DKO* mice. Increased MMP activity can lead to either increased plaque stability by facilitating SMC proliferation and migration or decreased plaque stability by increasing collagen degradation. It is thus plausible that increased local MMP activity might have promoted VSMC proliferation and migration resulting in a stable plaque with thicker fibrous cap in the *DKO* mice.

Although features of plaque stability are enhanced in both the genders, total aortic plaque burden was reduced in females, but not males, suggesting that plaque stability is gender-

independent, whereas plaque development is gender dependent in the *DKO* mice. However, it is not known whether female sex hormones regulate TRAF3IP2 expression. In a recent study, TRAF3IP2 has been shown to play a role in estrogen-deficient osteoporosis (9), suggesting that estrogen might regulate TRAF3IP2 expression by mechanisms not yet described. Further, deletion of TRAF6, a downstream target of TRAF3IP2, in endothelial cells resulted in reduced atherosclerosis in females but not males, further supporting our observations (31). A limitation of our study is that, though we have determined the effects of global deletion of *TRAF3IP2* on atherosclerotic plaque formation and composition, the contribution of TRAF3IP2 from individual cell types on the observed phenotype has not been determined.

In conclusion, we have demonstrated for the first time a causal role for TRAF3IP2 in atherosclerosis. *TRAF3IP2* gene deletion not only decreased plaque development, but also increased features of plaque stability in $ApoE^{-/-}$ mice. Interestingly, *TRAF3IP2* gene deletion failed to modulate systemic cholesterol levels. However, its lack of expression blunted proinflammatory cytokine, chemokine, adhesion molecule, and MMP expression. Thus, TRAF3IP2 could be a potential therapeutic target in atherosclerotic vascular diseases. Our future studies will determine whether gelatin nanoparticle or adeno-associated virus-mediated delivery of TRAF3IP2 siRNA or shRNA will blunt development of atherosclerosis and promote plaque stability in $ApoE^{-/-}$ mice fed a Western diet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Act1	activator of NF-κB
AP-1	activator protein-1
ADAM	A Disintegrin and metalloproteinase domain-containing protein
AGTR	angiotensin II receptor
AngII	angiotensin II
AOPPs	advanced oxidation protein products
ApoE	Apolipoprotein E
IKK	IκB kinase

CXCL1	chemokine (C-X-C motif), ligand 1
ECM	Extracellular matrix
HDL	High-density lipoprotein
ICAM1	intercellular adhesion molecule 1
IκB	inhibitory kappa B
IL	interleukin
JNK	c-Jun amino-terminal kinase
LDL	Low-density lipoprotein
MMP	matrix metalloproteinase
NF-κB	nuclear factor κB
Ox-LDL	Oxidized low-density lipoprotein
ROS	reactive oxygen species
RECK	Reversion-Inducing-Cysteine-Rich Protein with Kazal Motifs
ROS	reactive oxygen species
αSMA	alpha smooth muscle actin
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumor necrosis factor α
VCAM1	Vascular cell adhesion molecule
TRAF	TNF Receptor Associated Factor
TRAF3IP2	TRAF3-Interacting Protein 2
VLDL	Very low-density lipoprotein
VSMC	vascular smooth muscle cell

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Highlights

1. TRAF3IP2 gene deletion reduces atherosclerosis in female *ApoE*$^{-/-}$ mice.
2. TRAF3IP2 gene deletion decreases plaque necrotic core size in both genders.
3. TRAF3IP2 gene deletion increases features of plaque stability.
4. TRAF3IP2 gene deletion inhibits aortic proinflammatory and extracellular matrix genes.
5. TRAF3IP2 could be a potential therapeutic target in atherosclerotic vascular diseases.

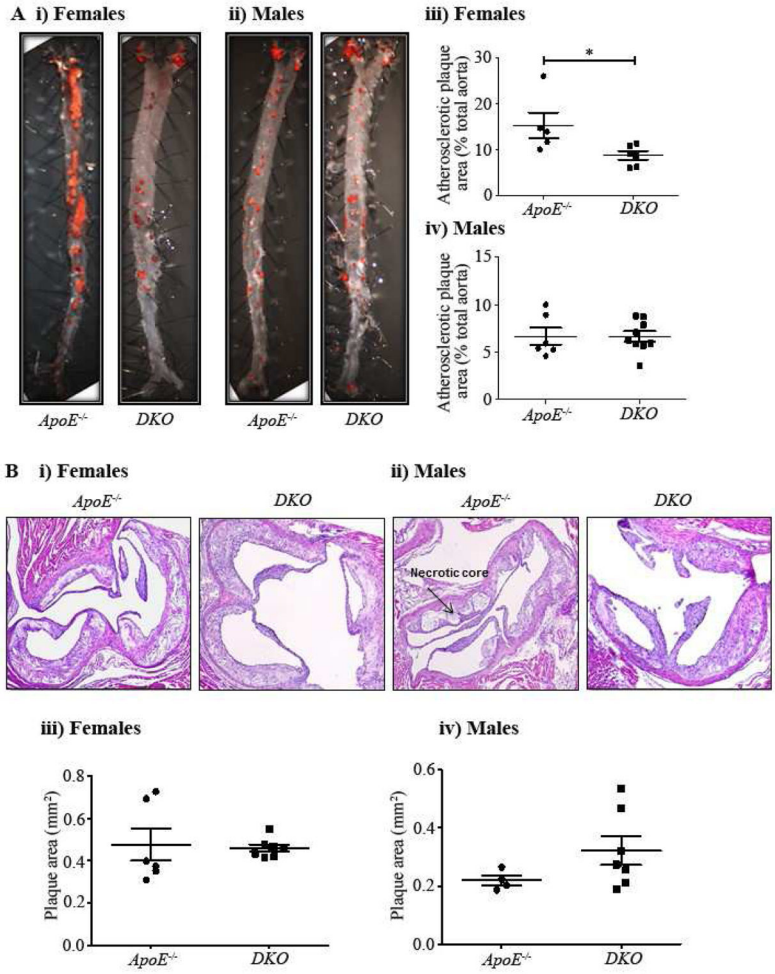

Fig. 1. Total aortic plaque burden and aortic root plaque cross-sectional area in *ApoE*$^{-/-}$ and DKO mice fed a Western diet

A(i), Representative Oil Red O-stained ***en face*** aortas from ***ApoE***$^{-/-}$ and ***DKO*** female mice. A(ii), Representative Oil Red O-stained *en face* aortas from *ApoE*$^{-/-}$ and *DKO* male mice. A(iii), Quantitative assessment of plaque area in *ApoE*$^{-/-}$ and *DKO* female mice. A(iv), Quantitative assessment of plaque area in *ApoE*$^{-/-}$ and *DKO* male mice. B(i), Representative aortic root cross sections from *ApoE*$^{-/-}$ and *DKO* female mice (magnification X100). B(ii). Representative aortic root cross sections from *ApoE*$^{-/-}$ and *DKO* male mice (magnification X100). B(iii). Quantitative assessment of plaque area in *ApoE*$^{-/-}$ and *DKO* female mice. B(iv). Quantitative assessment of plaque area in *ApoE*$^{-/-}$ and *DKO* male mice. Values are mean ± SEM. Statistical significance was calculated by Student's t-test (*p <0.05).

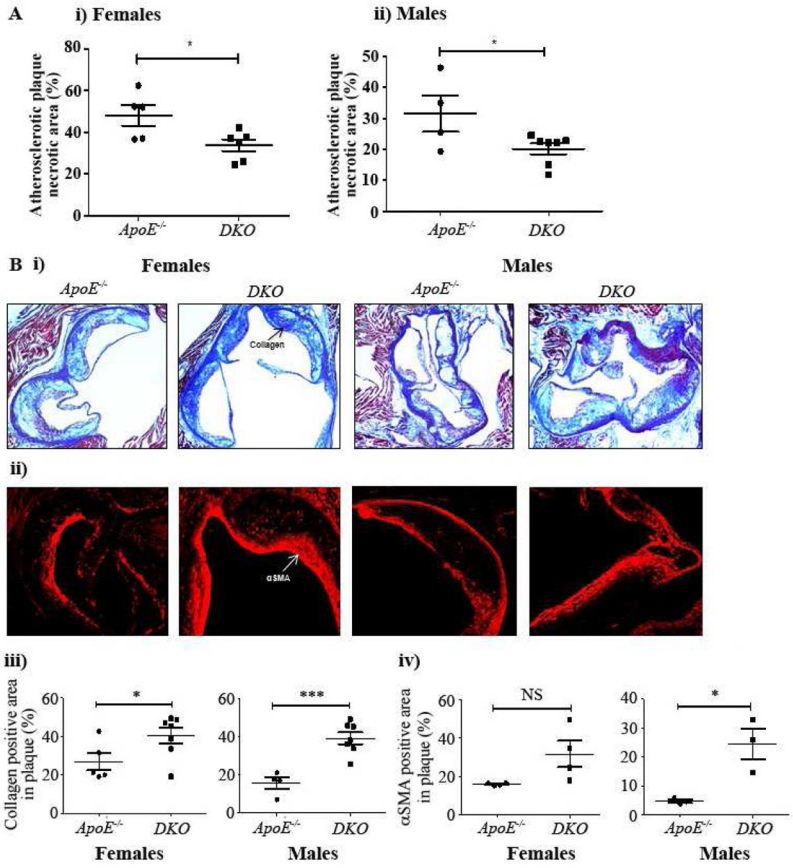

Fig. 2. Necrotic core, collagen-positive and smooth muscle cell-positive areas in aortic root valve plaques in *ApoE*$^{-/-}$ and DKO mice fed a Western diet

A(i), Quantitative assessment of the aortic root plaque necrotic area in *ApoE*$^{-/-}$ and *DKO* female mice. A (ii). Quantitative assessment of the aortic root plaque necrotic area in *ApoE*$^{-/-}$ and *DKO* male mice. B (i). Representative aortic root cross sections stained with Masson's trichrome from *ApoE*$^{-/-}$ and *DKO* female and male mice. B(ii), Representative aortic root cross sections for αSMA of *ApoE*$^{-/-}$ and *DKO* female and male mice (magnification X200). B(iii), Quantitative assessment of collagen- positive area in *ApoE*$^{-/-}$ and *DKO* female and male mice. B(iv), Quantitative assessment of α SMA-positive area in *ApoE*$^{-/-}$ and *DKO* female and male mice. Values are mean ± SEM. Statistical significance was calculated by Student's t-test (*p <0.05, *** p <0.001). NS, not significant.

Table 1

Plasma lipid profile of *ApoE*$^{-/-}$ and *DKO* mice fed a Western diet

	***ApoE*$^{-}$ female**	***DKO* female**	***ApoE*$^{-/-}$ male**	***DKO* male**
Triglycerides (mg/dL)	97.0±19.2	114.6±40.4	96.69±39.23	205.2±40.1
Total cholesterol (mg/dL)	1502±142	1748±127	2048±207	2070±183
VLDL/LDL cholesterol (mg/dL)	1189±152	1161±79.4	1292±99.0	1030±140
HDL cholesterol (mg/dL)	23.5±6.50	21.5±6.00	26.7±9.8	56.7±16.7

Values are represented in mean ± SEM; n=4–7 mice/group. *DKO,* double knockout mice (*TRAF3IP2*$^{-/-}$/*ApoE*$^{-/-}$). Comparisons were made between *ApoE*$^{-/-}$ and *DKO* mice belonging to the same gender. Statistical significance was calculated by Student's t-test.

Table 2

Gene expression in aortas from *ApoE*$^{-/-}$ and *DKO* mice fed a Western diet

Target gene	*ApoE*$^{-/-}$ female	*DKO* female	*ApoE*$^{-/-}$ male	*DKO* male
IL-6	1.00±0.45	1.37±0.80	1.00±0.44	0.08±0.04
IL-18	1.00±0.60	0.87±0.44	1.00±0.34	1.269±0.23
TNFα	1.00±0.64	0.18±0.13	1.00±0.60	0.30±0.06
ICAM1	1.00±0.25	0.28±0.18	1.00±0.23	0.15±0.04*
VCAM1	1.00±0.26	0.29±0.15	1.00±0.23	0.57±0.27
MCP1	1.00±0.25	0.99±0.72	1.00±0.05	0.73±0.33
CXCL1	1.00±0.09	0.32±0.17*	1.00±0.33	0.20±0.07*
AT1A	1.00±0.32	0.85±0.47	1.00±0.69	0.23±0.07
AT2	1.00±0.13	0.33±0.24	1.00±0.88	0.42±0.15
MMP2	1.00±0.33	0.64±0.40	1.00±0.42	0.31±0.12
MMP9	1.00±0.14	5.84±2.90	1.00±0.33	2.00±1.47
MMP14	1.00±0.53	0.52±0.38	1.00±0.33	0.66±0.16
TIMP1	1.00±0.11	0.53±0.57	1.00±0.10	0.40±0.07*
TRAF3IP2	1.00±0.34	-	1.00±0.23	-
RECK	1.00±0.40	0.73±0.31	1.00±0.13	0.25±0.05*
ADAM10	1.00±0.82	7.40±5.20	1.00±0.053	1.00±0.25
ADAM17	1.00±0.25	0.57±0.37	1.00±0.06	0.23±0.04*

IL-6, interleukin-6; IL-18, interleukin-18; TNFα, tumor necrosis factor α; ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule; AT1A, angiotensin II receptor, type 1a; AT2, angiotensin II receptor, type 2; MMP, matrix metalloproteinase; TIMP1, tissue inhibitor of matrix metalloproteinase 1; TRAF3IP2, TRAF3-Interacting Protein 2; RECK, reversion-inducing cysteine-rich protein with kazal motifs; ADAM, a disintegrin and metalloproteinase. 18S rRNA served as the endogenous invariant control, and relative gene expression in *ApoE*$^{-/-}$ female mice were taken as '1'. *DKO*, double knockout mice (*TRAF3IP2*$^{-/-}$/*ApoE*$^{-/-}$). Values are mean ± SEM (n = 3–5).

*indicates significance at $p<0.05$ when compared between *ApoE*$^{-/-}$ and *DKO* mice belonging to the same gender. Statistical significance was calculated by Student's t-test.