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STING, a cytosolic DNA sensor, plays a critical role in atherogenesis: a link between innate immunity and chronic inflammation caused by lifestyle-related diseases

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See page 4349 for the editorial comment for this article 'DNA damage and extranuclear DNA sensors: a dangerous duo in atherosclerosis', by D. Santovito and S. Steffens, https://doi.org/10.1093/eurheartj/ehab300.

Aims Lifestyle-related diseases promote atherosclerosis, a chronic inflammatory disease; however, the molecular mechanism remains largely unknown. Endogenous DNA fragments released under over-nutrient condition provoke sterile inflammation through the recognition by DNA sensors. Here, we investigated the role of stimulator of interferon genes (STING), a cytosolic DNA sensor, in atherogenesis.

Methods and results Apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice fed a western-type diet (WTD), a hypercholesterolaemic mouse model, showed higher STING expression and markers for DNA damage such as γH2AX, p53, and single-stranded DNA (ssDNA) accumulation in macrophages in the aorta compared with wild-type (WT) mice. The level of cGAMP, a STING agonist, in the aorta was higher in *Apoe*$^{-/-}$ mice. Genetic deletion of *Sting* in *Apoe*$^{-/-}$ mice reduced atherosclerotic lesions in the aortic arch, lipid, and macrophage accumulation in plaques, and inflammatory molecule expression in the aorta compared with the control. Pharmacological blockade of STING using a specific inhibitor, C-176, ameliorated atherogenesis in *Apoe*$^{-/-}$ mice. In contrast, bone marrow-specific STING expression in *Apoe*$^{-/-}$ mice stimulated atherogenesis. Expression or deletion of STING did not affect metabolic parameters and blood pressure. *In vitro* studies revealed that STING activation by cGAMP or mitochondrial DNA accelerated inflammatory molecule expression (e.g. TNF-α or IFN-β) in mouse and human macrophages. Activation of nuclear factor-κB and TANK binding kinase 1 was involved in STING-associated vascular inflammation and macrophage activation. Furthermore, human atherosclerotic lesions in the carotid arteries expressed STING and cGAMP.

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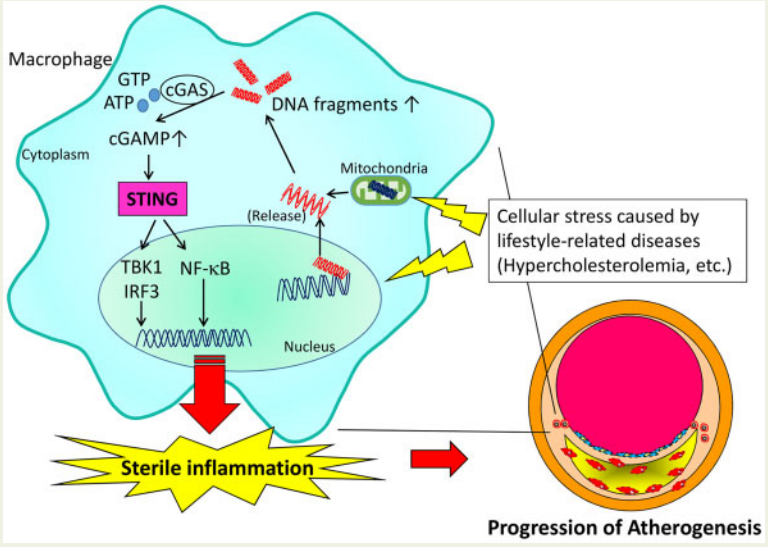
† These authors contributed equally to this study.

Conclusion Stimulator of interferon genes stimulates pro-inflammatory activation of macrophages, leading to the development of atherosclerosis. Stimulator of interferon genes signalling may serve as a potential therapeutic target for atherosclerosis.

Graphical Abstract



STING signalling, originally associated with innate immune system, may provide a novel mechanism of atherogenesis by linking lifestyle-related diseases to chronic inflammatory disease and serve as a potential therapeutic target for atherosclerosis.

Keywords STING • DNA • Inflammation • Macrophage • Atherosclerosis

Translational perspective

Lifestyle-related diseases cause vascular inflammation and subsequent atherosclerosis; however, underlying mechanisms remain largely unknown. Accumulating evidence suggested that endogenous DNA fragments released under over-nutrient condition provoke sterile inflammation. Here, we demonstrate a pivotal role of stimulator of interferon genes (STING), a cytosolic DNA sensor, in atherogenesis. In hypercholesterolaemic mice, DNA damage and cGAMP, a direct STING agonist, increased in the aorta. Genetic deletion or pharmacological blockade of STING ameliorated atherogenesis. Stimulator of interferon genes activation promoted inflammatory molecule expression in human and mouse macrophages. Furthermore, human atherosclerotic lesions also expressed STING and cGAMP. Stimulator of interferon genes signalling, which links lifestyle-related diseases to chronic inflammatory disease, may provide a novel mechanism of atherogenesis and serve as a potential therapeutic target for atherosclerosis.

Introduction

Chronic inflammation in the vasculature causes atherosclerosis[1]; however, underlying molecular mechanisms remain not fully understood. Recent advances in immunology have dissected molecular pathways involved in the innate immune system that induce and promote inflammatory responses in the vascular wall.[2] Pattern recognition receptors (PPRs) such as Toll-like receptors (TLRs) on immune cells including macrophages contribute to self-defence by activation of inflammation against molecules typically associated with pathogens.[3] Besides, emerging evidence has revealed that PPRs have roles in the pathogenesis of various diseases by activating sterile inflammation in response to various endogenous ligands.[4,5] Endogenous DNA fragments released from or accumulated in host cells function as important endogenous ligands, though DNA is indispensable for life.[6] Recent studies have revealed that physiological and pathological

processes in lifestyle-associated diseases such as diabetes, hypercholesterolaemia, and ageing, cause degradation of cells and subsequent damage of both nuclear and mitochondrial DNA,[7–11] which results in the release and/or accumulation of DNA fragments.[12–14] This DNA damage contributes to the initiation and progression of inflammatory diseases including atherosclerosis.[15–21]

Stimulator of interferon genes (STING) was originally known as a cytosolic DNA sensor that activates the innate immune system against pathogens.[22,23] Stimulator of interferon genes is activated by cyclic GMP-AMP (cGAMP), an intracellular second messenger that is synthesized in the interaction of cyclic GMP-AMP synthase (cGAS) and cytosolic DNA fragments.[24,25] Subsequently, STING activates transcription factors, interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB), via TANK binding kinase (TBK)1 and IκB kinase (IKK), respectively, which produces type I interferons (IFNs) and cytokines after translocation into the nucleus.[26,27] Recent studies suggested that endogenous DNA released and/or leaked from the nucleus or mitochondria into the cytoplasm also activates this pathway, and a link between STING and the pathogenesis of various diseases such as tumourgenesis, vasculitis, autoimmune diseases, and lifestyle-related diseases.[26–32] However, the role of STING signalling in atherogenesis remains obscure.

In this study, we hypothesized that STING signalling promotes vascular inflammation and atherogenesis. To address our hypothesis, we performed *in vivo* studies using apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice and *Apoe*$^{-/-}$ mice, which genetically lack *Sting* (*Sting*$^{-/-}$*Apoe*$^{-/-}$mice), and *in vitro* experiments using macrophages because pro-inflammatory activation of macrophages is an essential feature of the development of atherogenesis.[33] Furthermore, we examined the expression of cGAMP, a STING agonist, in atherosclerotic lesions in mice and humans to obtain clinically translatable evidence showing the contribution of STING signalling to atherogenesis. The results of our study proposed a novel mechanism involving STING, a player in the innate immune system, in vascular inflammation and atherogenesis, and suggested the potential of a therapeutic strategy targeting STING for atherosclerotic diseases.

Methods

Methods are provided in detail in the Supplementary material online.

Animal study

All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. The protocol was reviewed and approved by the Animal Care and Use Committee of Tokushima University.

Analysis of human atherosclerotic lesions

Atherosclerotic lesions collected by carotid endarterectomy were used in this study. All patients underwent carotid endarterectomy at the Department of Neurosurgery of Tokushima University Hospital because of moderate to severe symptomatic stenosis or severe non-symptomatic stenosis in the carotid artery. The Institutional Review Board of Tokushima University Hospital approved the study protocol. Macroscopically normal carotid arteries provided by ProteoGenex, Inc. were used as the control.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Measurement of cGAMP in atherosclerotic lesions was performed by LC-MS/MS. For further details, please refer to the Supplementary material online.

Statistical analysis

All numerical values are expressed as mean ± SD. The Shapiro–Wilk normality test was used to assess whether variables had a normal distribution. Comparison of parameters between two groups was performed with unpaired Student's *t*-test when data showed a normal distribution or with Mann–Whitney *U* test when data did not show a normal distribution. For human macrophage experiments, Wilcoxon signed-rank test was used to perform paired comparison of the expression of inflammatory molecules between non-treatment and treatment conditions, because data did not show a normal distribution. A *P*-value of <0.05 was considered statistically significant.

Results

Stimulator of interferon genes expression and DNA damage increased in atherosclerotic aorta

First, we examined the expression of STING and cGAMP in atherosclerotic lesions in western-type diet (WTD) fed *Apoe*$^{-/-}$ mice, a mouse model for hypercholesterolaemia-associated atherosclerosis. Stimulator of interferon genes expression increased in the aorta of *Apoe*$^{-/-}$ mice compared with wild-type (WT) mice (*Figure 1A* and *B*). Stimulator of interferon genes expression co-localized with a marker for macrophages, Mac3, in atherosclerotic plaques (*Figure 1C*). The aorta obtained from *Apoe*$^{-/-}$ mice showed features of DNA damage such as higher expression of γH2AX, p53, and accumulation of single-stranded DNA (ssDNA) in macrophages (*Figure 1D* and *E*). We further examined whether DNA damage associates with the increase in cGAMP. The results of highly sensitive LS-MS/MS analysis clearly demonstrated that cGAMP level in the aorta was higher in *Apoe*$^{-/-}$ mice compared with WT mice ($P = 0.029$, *Figure 1F* and *G*).

Genetic deletion of stimulator of interferon genes ameliorated atherogenesis in *Apoe*$^{-/-}$ mice

To investigate the role of STING in atherogenesis, we compared atherosclerotic lesion progression in the aortic arch between *Apoe*$^{-/-}$ mice and *Sting*$^{-/-}$*Apoe*$^{-/-}$mice after 20 weeks of WTD feeding. The absence of STING expression in *Sting*$^{-/-}$*Apoe*$^{-/-}$mice was confirmed in the aorta and leucocytes (Supplementary material online, *Figure S1*). *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice demonstrated a significant reduction in atherosclerotic lesion development in the aortic arch ($P = 0.17 \times 10^{-4}$) and the aortic root ($P = 0.017$), accompanied with reduction of lipid deposition, and macrophage accumulation as determined by Mac3 expression ($P = 0.013$), in atherosclerotic lesions (*Figure 2A–C*), compared with *Apoe*$^{-/-}$ mice. In addition, the expression of anti-inflammatory macrophage marker, CD206, was higher in *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice (Supplementary material online, *Figure S2*). Systemic

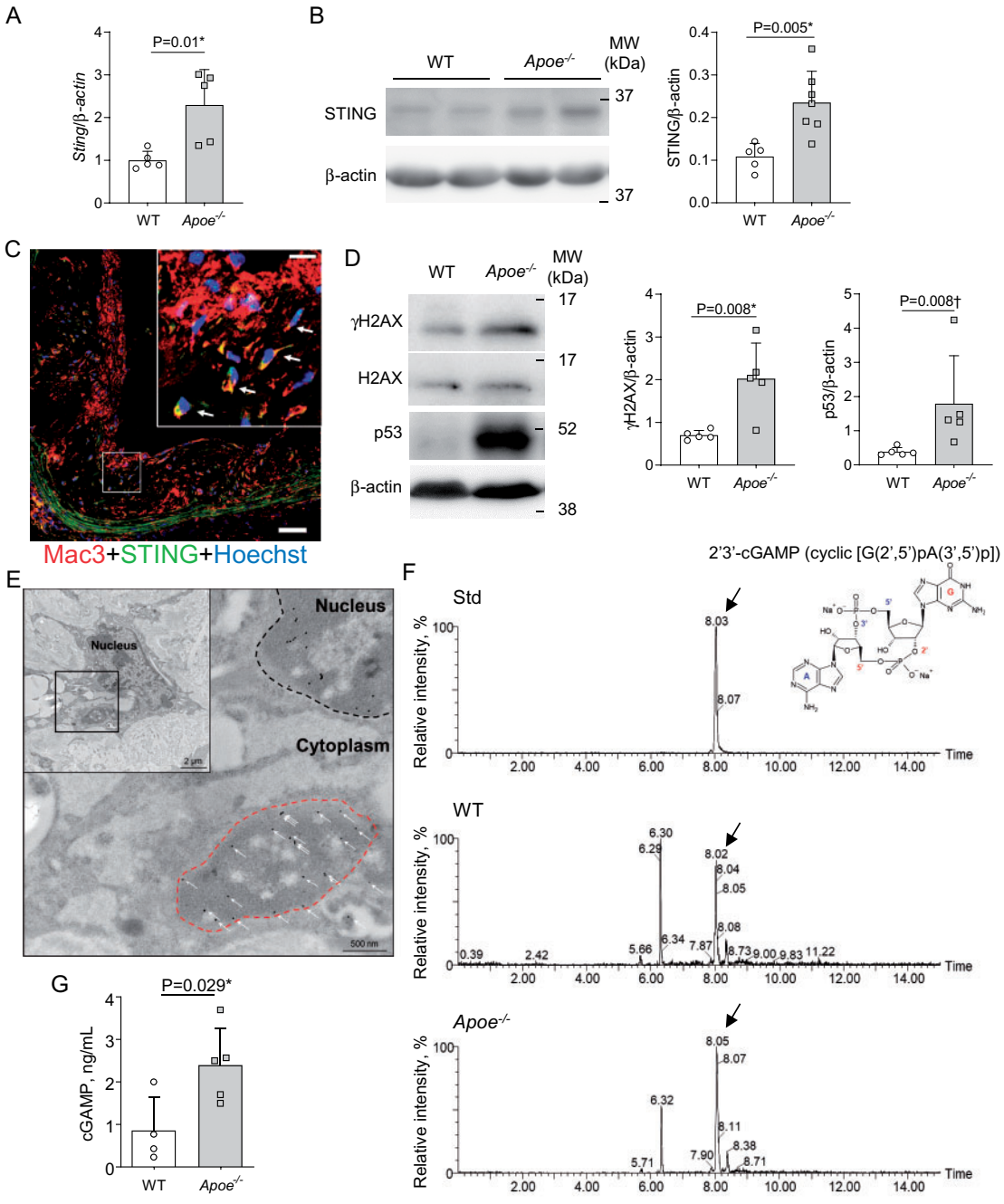


Figure 1 Stimulator of interferon genes expression and DNA damage in atherosclerotic lesions in mice. (*A* and *B*) qPCR analysis (A) and western blot analysis (B) indicated that western-type diet-fed *Apoe*$^{-/-}$ mice had higher stimulator of interferon genes expression in the aorta compared with wild-type mice (n = 5–7). (*C*) Immunofluorescence staining of the aortic root of *Apoe*$^{-/-}$ mice demonstrated co-localization of stimulator of interferon genes (green) and a macrophage marker, Mac3 (red) (arrows in inset) in plaques. Scale bar: 50 μm (inset, 10 μm). (*D*) Western blot analysis demonstrated higher γH2AX and p53 expression in the aorta of *Apoe*$^{-/-}$ mice (n = 5). (*E*) Representative immunogold staining against single-stranded DNA revealing accumulation of gold particles (15 nm) in the cytoplasm of macrophages (white arrows) in atherosclerotic lesion in *Apoe*$^{-/-}$ mouse, indicating single-stranded DNA accumulation in the macrophage cytoplasm (n = 3). Red dashed line represents an aggregate accumulation of gold particles (scale bar: 500 nm). Inset: lower magnification (scale bar: 2 μm). (*F* and *G*) Representative chromatogram of mouse aorta indicating cGAMP separation (*F*). The retention time was 8.03 min for standard, 8.02 min for wild-type mice, and 8.05 min for *Apoe*$^{-/-}$ mice. Arrows indicated the peak for cGAMP. cGAMP was quantified using calibration curve. The standard calibration curve of cGAMP concentration was established by linear regression analysis between cGAMP concentration (0–100 ng/mL) and the peak area. cGAMP concentration in the aorta was higher in *Apoe*$^{-/-}$ mice compared with wild-type mice (n = 4–5) (*G*). MW, molecular weight; Std, standard. *Analysed with Student's t-test. †Analysed with Mann–Whitney U test. All values are mean ± SD.

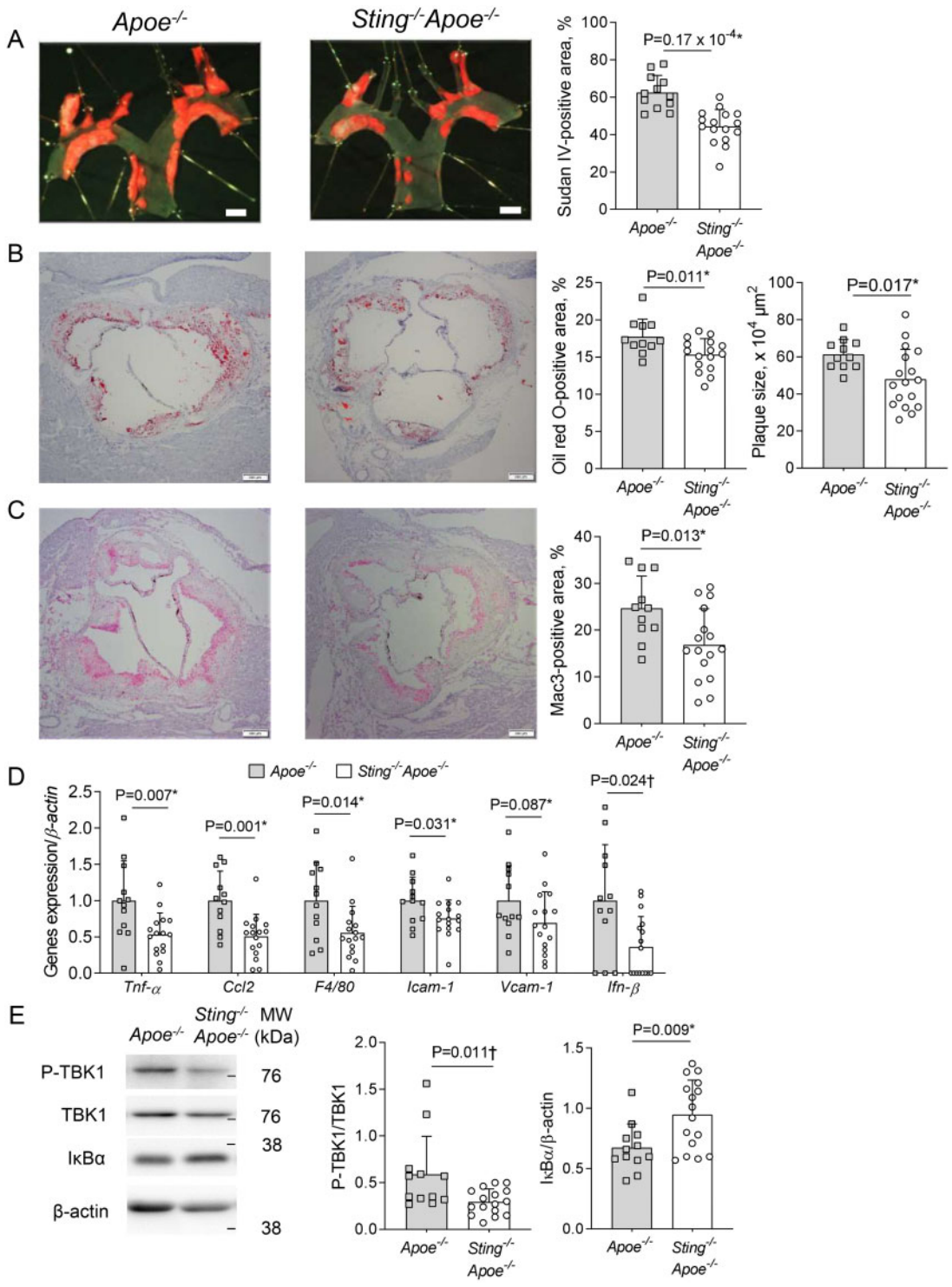


Figure 2 Genetic deletion of stimulator of interferon genes attenuated atherogenesis in *Apoe*$^{-/-}$ mice. (*A*) En-face Sudan IV staining of the aortic arch demonstrated that *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice had reduced atherosclerotic lesions compared with *Apoe*$^{-/-}$ mice after 20 weeks of western-type diet feeding (n = 12–16). Scale bar: 1 mm. (*B* and *C*) Oil red O staining (*B*) and immunostaining against Mac3 (*C*) in atherosclerotic lesions in the aortic root demonstrated that *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice had smaller plaques, and reduced lipid deposition and macrophage accumulation in the plaques compared with *Apoe*$^{-/-}$ mice (n = 11–16). Scale bar: 200 µm. (*D*) qPCR analysis of the abdominal aorta revealed that genetic deletion of *Sting* decreased the expression of inflammatory molecules (n = 12–16). (*E*) Western blot analysis demonstrated that phosphorylation of TBK1 and degradation of IκBα were reduced in the aorta of *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice compared with that of *Apoe*$^{-/-}$ mice (n = 12–16). *Analysed with Student's t-test. †Analysed with Mann–Whitney U test. All values are mean ± SD.

deletion of *Sting* reduced the expression of inflammatory molecules such as *Tnf-α* ($P = 0.007$), *Ccl2* ($P = 0.001$), *Ifn-β* ($P = 0.024$), and macrophage marker, *F4/80* ($P = 0.014$) in the aorta (*Figure 2D*). *Sting* deletion also reduced the phosphorylation of TBK1 and attenuated the degradation of IκBα in the atherosclerotic aorta, suggesting inhibition of TBK1 signalling and NF-κB signalling ($P = 0.011$ and $P = 0.009$, respectively) (*Figure 2E*). Bodyweight of *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice was smaller than that of *Apoe*$^{-/-}$ mice; however, metabolic parameters and blood pressure were comparable between the two mouse strains (Supplementary material online, *Table S1*). These results indicated that STING signalling plays a pivotal role in vascular inflammation and atherogenesis in *Apoe*$^{-/-}$ mice fed a WTD. Genetic deletion of STING also attenuated atherogenesis in normal chow-fed *Apoe*$^{-/-}$ mice, though it elevated plasma triglyceride level (Supplementary material online, *Figure S3* and *Table S2*).

Bone marrow-specific expression of stimulator of interferon genes promoted atherogenesis in *Apoe*$^{-/-}$ mice

Immune cells such as macrophages are major populations that express STING. We, therefore, performed bone marrow (BM) transplantation to investigate the role of STING in BM cells in atherogenesis (Supplementary material online, *Figure S4*). Restoration of STING in BM in *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice increased atherosclerotic lesions in the aortic arch ($P = 0.017$) and in the aortic root ($P = 0.005$), and lipid deposition and inflammatory features in atherosclerotic plaques compared with BM-chimeric mice, which do not have STING in their body (*Figure 3A–C*, Supplementary material online, *Figure S5*). The results of quantitative polymerase chain reaction (qPCR) demonstrated that BM-specific STING expression in *Apoe*$^{-/-}$ mice promoted the expression of inflammatory molecules in the abdominal aorta compared with control BM-chimeric mice (*Figure 3D*). Bone marrow-specific STING expression also increased the phosphorylation of TBK1 in the aorta (*Figure 3E*). There were no significant differences in metabolic parameters and blood pressure (Supplementary material online, *Table S3*). These results indicated that STING in BM cells contributes to the development of vascular inflammation and atherogenesis.

Stimulator of interferon genes signal promoted pro-inflammatory activation of macrophages

Next, we examined the roles of STING in pro-inflammatory activation of macrophages. Treatments with cGAMP, a specific STING agonist, significantly promoted the expression of inflammatory molecules (e.g. *Tnf-α, Ccl2*, and *Ifn-β*) in *Apoe*$^{-/-}$ macrophages, but not in *Sting*$^{-/-}$*Apoe*$^{-/-}$ macrophages (*Figure 4A*, Supplementary material online, *Figure S6A*). The results of western blot analysis showed that cGAMP activated both TBK1 signalling and NF-κB signalling in *Apoe*$^{-/-}$ macrophages (*Figure 4B*). These results suggested that STING signalling accelerated pro-inflammatory activation of macrophages. In addition to cGAMP, we stimulated macrophages with mitochondrial DNA (mtDNA) because cGAMP is generated from DNA fragments in the cytosol.[24,25] As observed in cGAMP treatment, mtDNA promoted expression of inflammatory molecules and activated both TBK1 signalling and NF-κB signalling more effectively in *Apoe*$^{-/-}$ macrophages than in *Sting*$^{-/-}$*Apoe*$^{-/-}$ macrophages (*Figure 4C* and *D*, Supplementary material online, *Figure S6B*). These results indicated that mtDNA activated macrophages at least partially via STING signalling.

Inhibition of stimulator of interferon genes signalling attenuated pro-inflammatory activation of macrophages

We further investigated the effect of STING inhibition on macrophage activation. C-176, a specific STING inhibitor, or amlexanox, a TBK1 inhibitor, ameliorated cGAMP-induced expression of inflammatory molecules such as *Tnf-α, Ccl2*, and *Ifn-β* (*Figure 5A* and *B*). These results suggested that the TBK1 pathway participates in STING-mediated inflammatory responses in macrophages at least in part. Similarly, C-176 partially suppressed expression of these inflammatory molecules induced by mtDNA in *Apoe*$^{-/-}$ macrophages (*Figure 5C*), while amlexanox reduced only *Ifn-β* expression but not other inflammatory molecules such as *Tnf-α* under stimulation with mtDNA (*Figure 5D*). The results of Enzyme-linked immunosorbent assay (ELISA) that used culture medium obtained from macrophages confirmed these results (*Figure 5E* and *F*). Additionally, in this study, we confirmed the role of STING signalling in macrophage activation by using other types of macrophage such as RAW264.7 cells, residential peritoneal macrophages, and BM-derived macrophages (Supplementary material online, *Figures S7–S9*). These results suggested that STING and its downstream molecule, TBK1, partially participate in mtDNA-induced macrophage activation.

Inhibition of stimulator of interferon genes ameliorated atherogenesis in *Apoe*$^{-/-}$ mice

To investigate whether inhibition of STING attenuates atherogenesis, we administered C-176 to *Apoe*$^{-/-}$ mice fed WTD. After 12 weeks of treatment, C-176 significantly reduced atherosclerotic lesions in the aortic arch ($P = 0.025$) and in the aortic root ($P = 0.031$), and lipid deposition in atherosclerotic plaques ($P = 0.006$) compared with vehicle (*Figure 6A* and *B*). However, in this study, we did not observe significant reduction of macrophage accumulation in atherosclerotic lesions and inflammatory features in the treatment group compared with the vehicle group (*Figure 6C*, Supplementary material online, *Figure S10*). However, C-176 decreased TBK1 phosphorylation in the aorta, suggesting inhibition of STING signalling at least partially (*Figure 6D*). C-176 did not affect metabolic parameters and blood pressure in *Apoe*$^{-/-}$ mice (Supplementary material online, *Table S4*). These results suggested the potential of STING signalling as a therapeutic target of atherosclerosis.

Stimulator of interferon genes signalling in human atherosclerotic lesions

Finally, we examined the clinical significance of STING signalling in human atherosclerotic lesions obtained by carotid endarterectomy. Patients' background and information of samples obtained from a

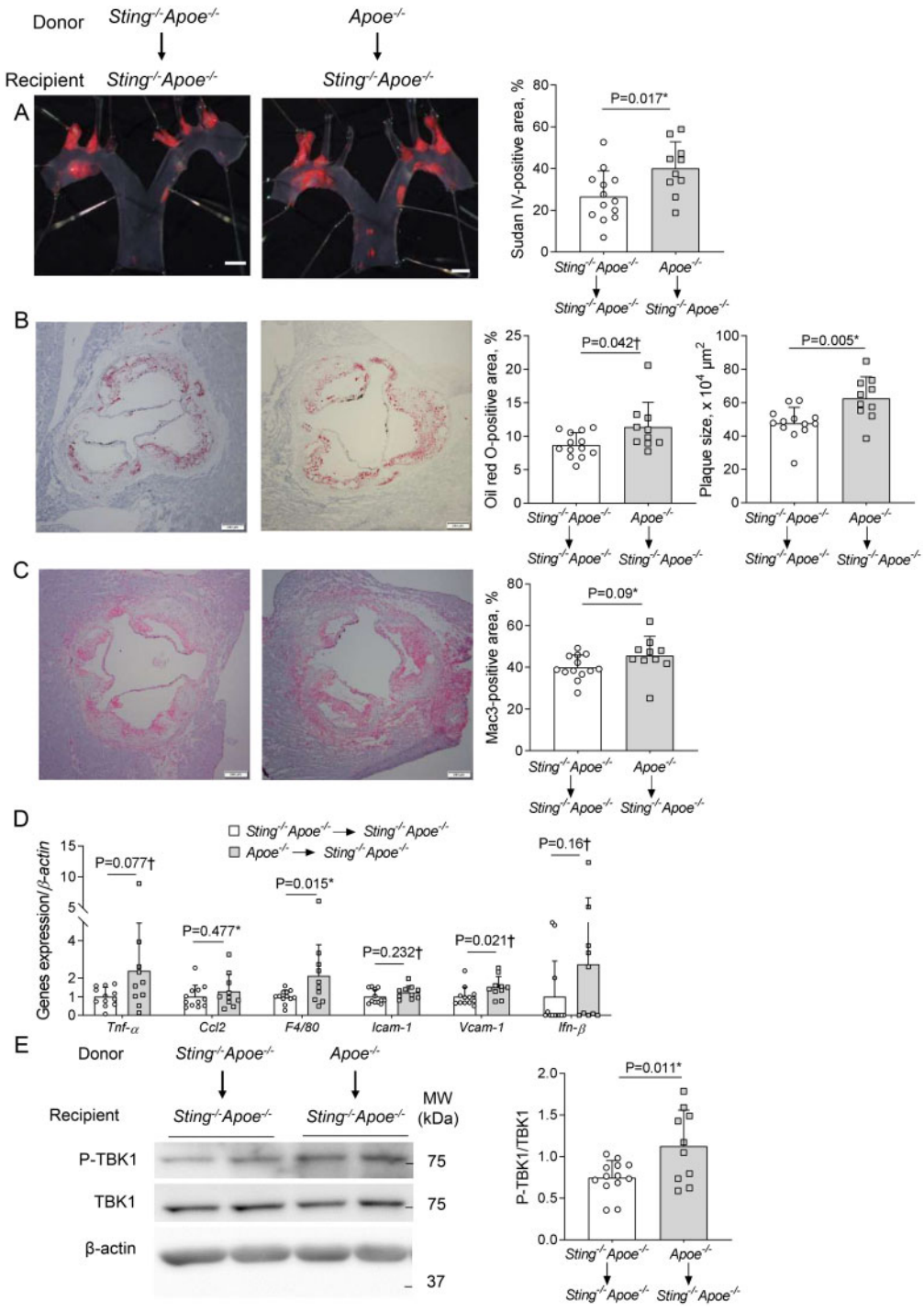


Figure 3 Bone marrow-specific expression of stimulator of interferon genes promoted atherogenesis in *Apoe*$^{-/-}$ mice. (*A*) En-face Sudan IV staining of the aortic arch demonstrated that bone marrow-specific expression of stimulator of interferon genes in *Apoe*$^{-/-}$ mice accelerated atherosclerotic lesion progression in the aortic arch compared with control bone marrow chimeric mice. Scale bar: 1 mm. (*B*) Oil red O staining in the aortic root demonstrated that bone marrow-specific stimulator of interferon genes expression in *Apoe*$^{-/-}$ mice increased plaque size and lipid deposition in atherosclerotic plaques. Scale bar: 200 µm. (*C*) Immunostaining against Mac3 demonstrated that bone marrow-specific stimulator of interferon genes expression in *Apoe*$^{-/-}$ mice increased macrophage accumulation in atherosclerotic plaques, although the difference was not significant. Scale bar: 200 µm. (*D*) qPCR analysis of the abdominal aorta revealed that bone marrow-specific stimulator of interferon genes expression in *Apoe*$^{-/-}$ mice increased inflammatory molecule expression in the aorta. (*E*) Western blot analysis demonstrated that bone marrow-specific stimulator of interferon genes expression in *Apoe*$^{-/-}$ mice increased phosphorylation of TBK1 in the aorta. n = 10–13. *Analysed with Student's t-test. †Analysed with Mann–Whitney U test. All values are mean ± SD.

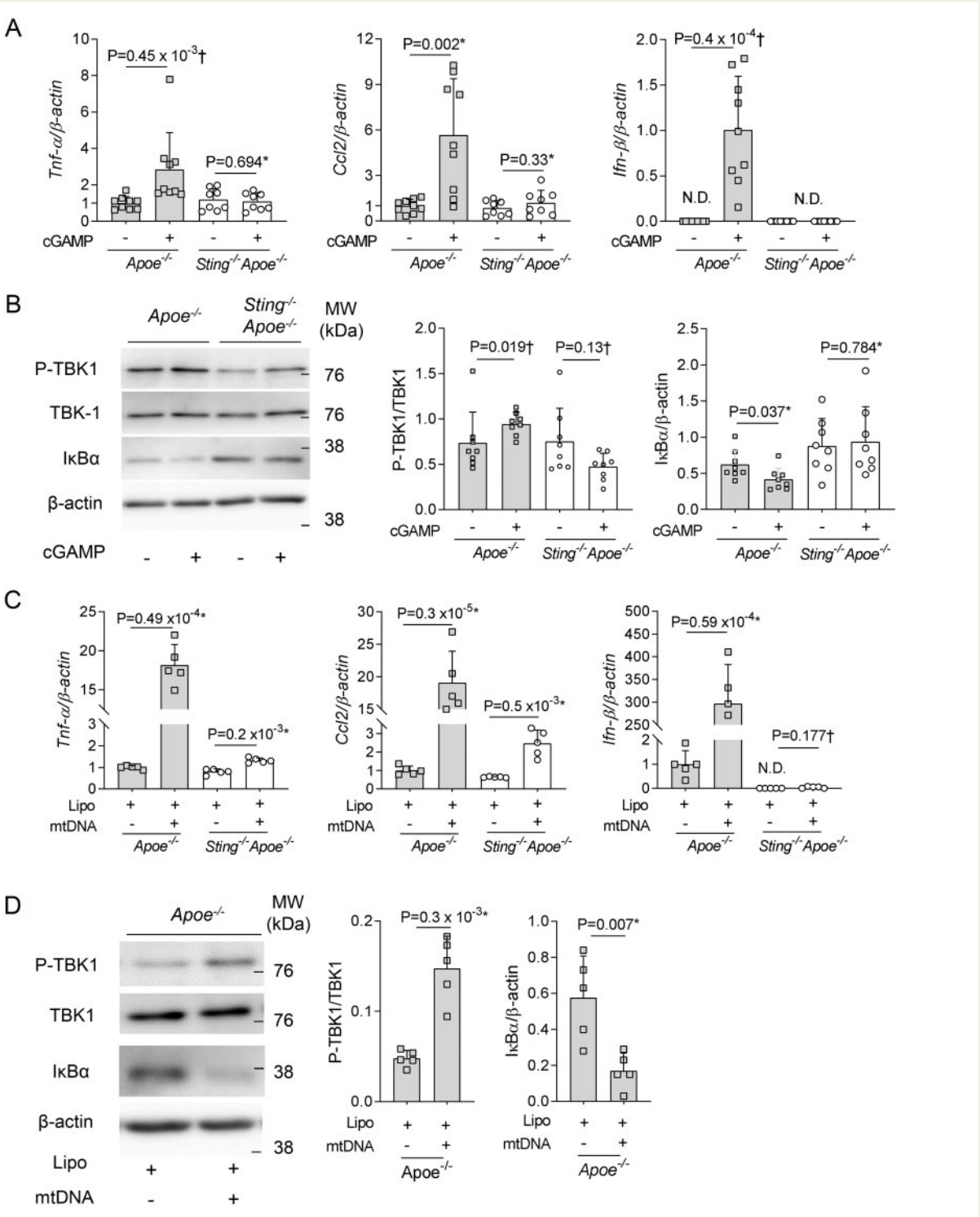


Figure 4 Stimulator of interferon genes signalling promoted macrophage activation. (*A*) qPCR analysis indicated that cGAMP, a specific stimulator of interferon genes agonist, increased the expression of inflammatory molecules such as *Tnf-α*, *Ccl2*, and *Ifn-β* in peritoneal macrophages obtained from *Apoe*$^{-/-}$ mice more effectively than in macrophages obtained from *Sting*$^{-/-}$*Apoe*$^{-/-}$mice (n = 8–9). (*B*) Western blot analysis demonstrated that cGAMP significantly promoted the phosphorylation of TBK1 and increased the degradation of IκBα in *Apoe*$^{-/-}$macrophages more effectively than in *Sting*$^{-/-}$*Apoe*$^{-/-}$ macrophages (n = 8). (*C*) qPCR analysis indicated that mtDNA increased the expression of inflammatory molecules such as *Tnf-α*, *Ccl2*, and *Ifn-β* in peritoneal macrophages obtained from *Apoe*$^{-/-}$mice more effectively than in macrophages obtained from *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice (n = 5). (*D*) Western blot analysis demonstrated that mtDNA significantly promoted the phosphorylation of TBK1 and the degradation of IκBα in *Apoe*$^{-/-}$ macrophages (n = 5). *Analysed with Student's t-test. †Analysed with Mann–Whitney U test. All values are mean ± SD.

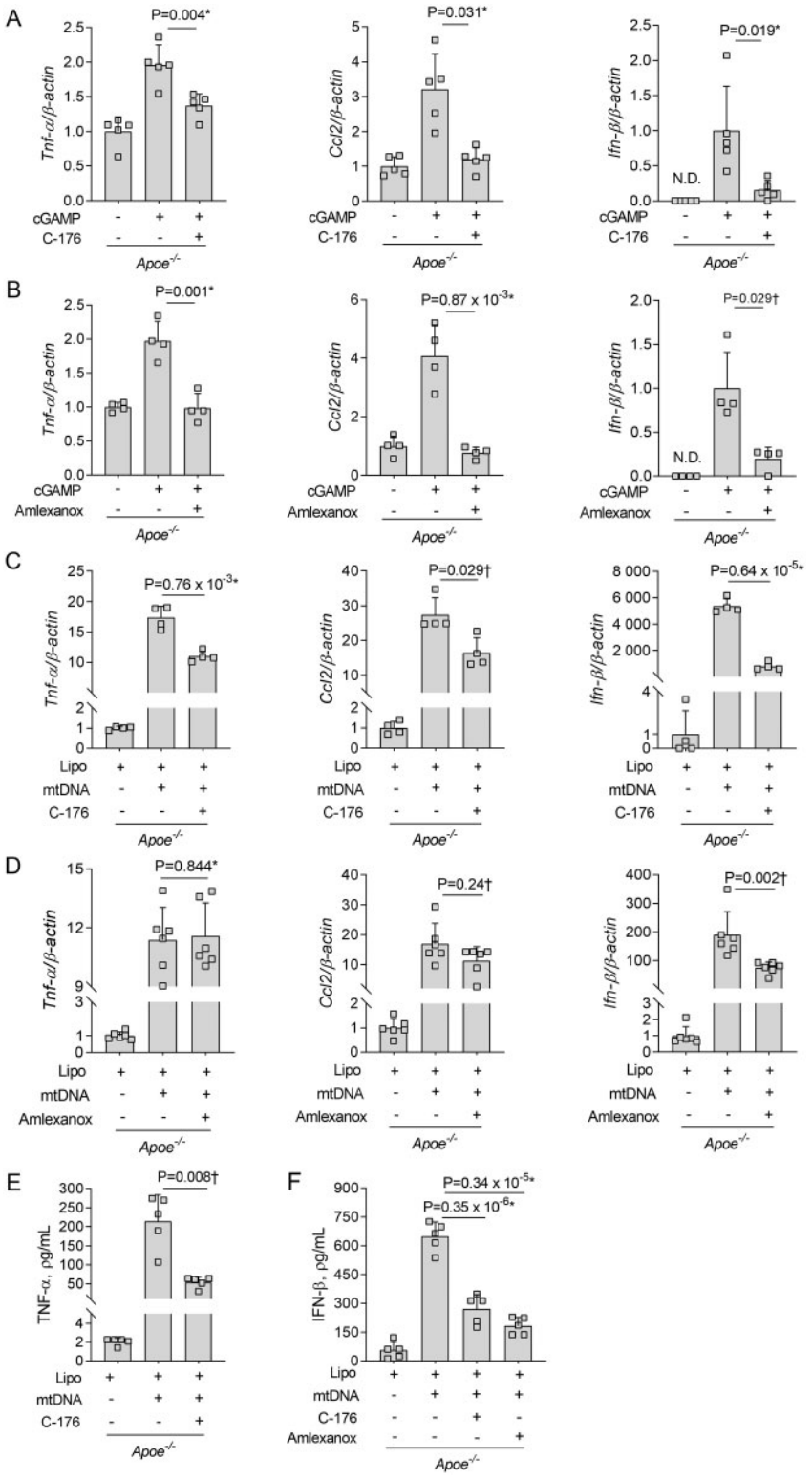


Figure 5 Inhibition of stimulator of interferon genes attenuated macrophage activation. (*A* and *B*) qPCR analysis indicated that a stimulator of interferon genes inhibitor, C-176, (*A*) or a TBK1 inhibitor, amlexanox, (*B*) ameliorated the expression of inflammatory molecules in *Apoe*$^{-/-}$macrophages, which were promoted by cGAMP (n = 4–5). (*C* and *D*) qPCR analysis indicated that C-176 (*C*) ameliorated the expression of inflammatory molecules in *Apoe*$^{-/-}$macrophages, which were promoted by mtDNA, although amlexanox (*D*) ameliorated only *Ifn-β* expression (n = 4–6). (*E* and *F*) The results of ELISA indicated that C-176 or amlexanox ameliorated the secretion of TNF-α (*E*) and IFN-β (*F*) from macrophages in response to mtDNA (n = 5). *Analysed with Student's t-test. †Analysed with Mann–Whitney U test. All values are mean ± SD.

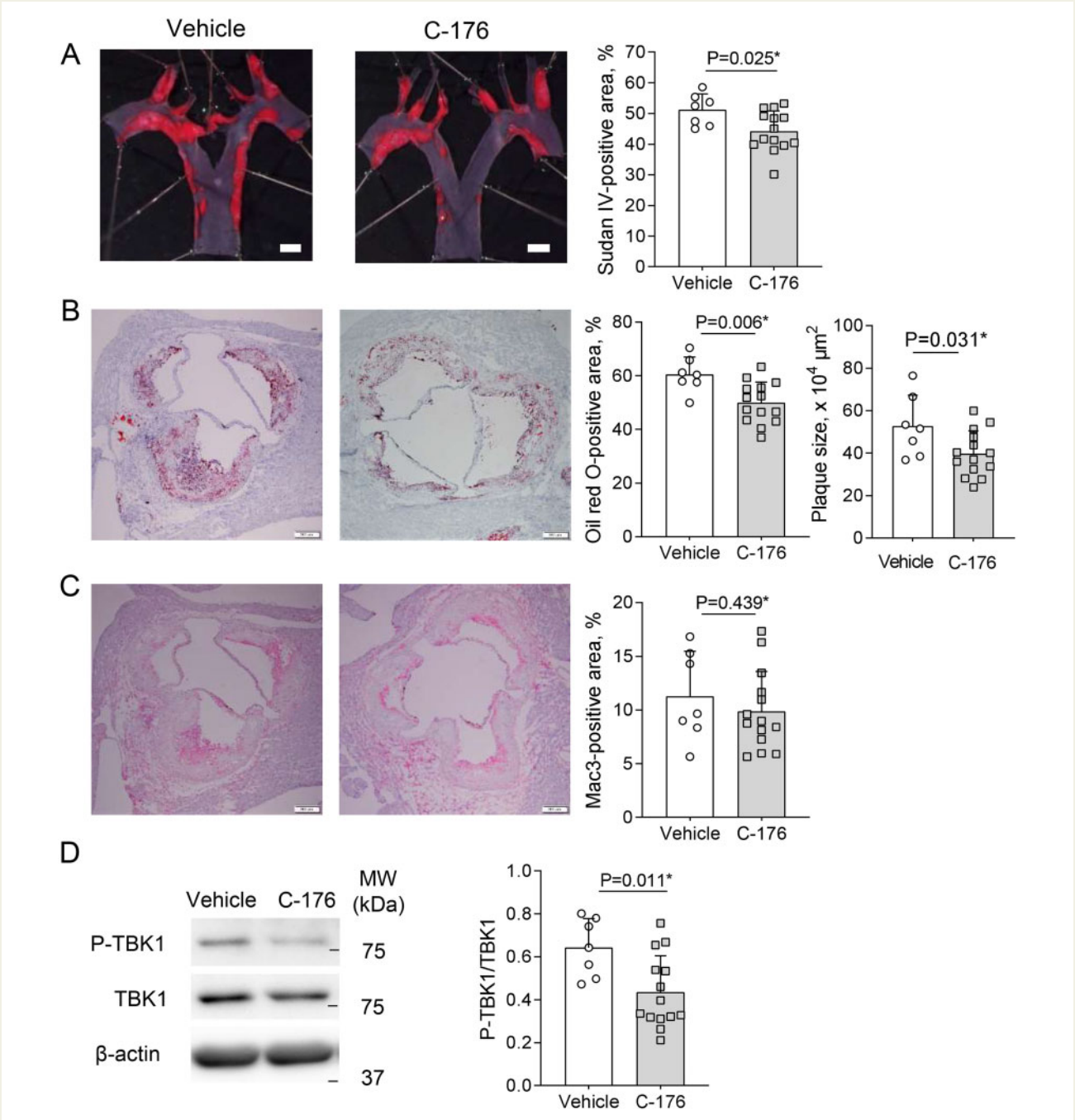


Figure 6 Pharmacological blockade of stimulator of interferon genes attenuated atherogenesis in *Apoe*$^{-/-}$ mice. (*A*) En-face Sudan IV staining of the aortic arch showed that administration of C-176, a stimulator of interferon genes inhibitor, reduced atherosclerotic lesion development in *Apoe*$^{-/-}$ mice compared with vehicle. Scale bar: 1 mm. (*B*) Oil red O staining demonstrated that C-176 reduced plaque size and lipid deposition in atherosclerotic plaques in *Apoe*$^{-/-}$ mice. Scale bar: 200 μm. (*C*) There was no difference in macrophage accumulation as determined by Mac3 staining between treated and control groups. Scale bar: 200 μm. (*D*) Western blot analysis demonstrated that C-176 decreased the phosphorylation of TBK1 in *Apoe*$^{-/-}$ mice. $n = 7–14$. *Analysed with Student's *t*-test. †Analysed with Mann–Whitney *U* test. All values are mean ± SD.

tissue bank are summarized in Supplementary material online, *Tables S5* and *S6*, respectively. Histological analysis demonstrated the expression of STING in human atherosclerotic lesions (*Figure 7A*). The expression of STING was significantly higher in atherosclerotic lesions ($n = 5$) compared with macroscopically normal carotid arteries obtained from a tissue bank ($n = 4$) (*Figure 7B* and *C*). Furthermore, LC-MS/MS analysis demonstrated cGAMP expression in human atherosclerotic lesions ($n = 5$, 1.79 ± 0.47 ng/mL). On the

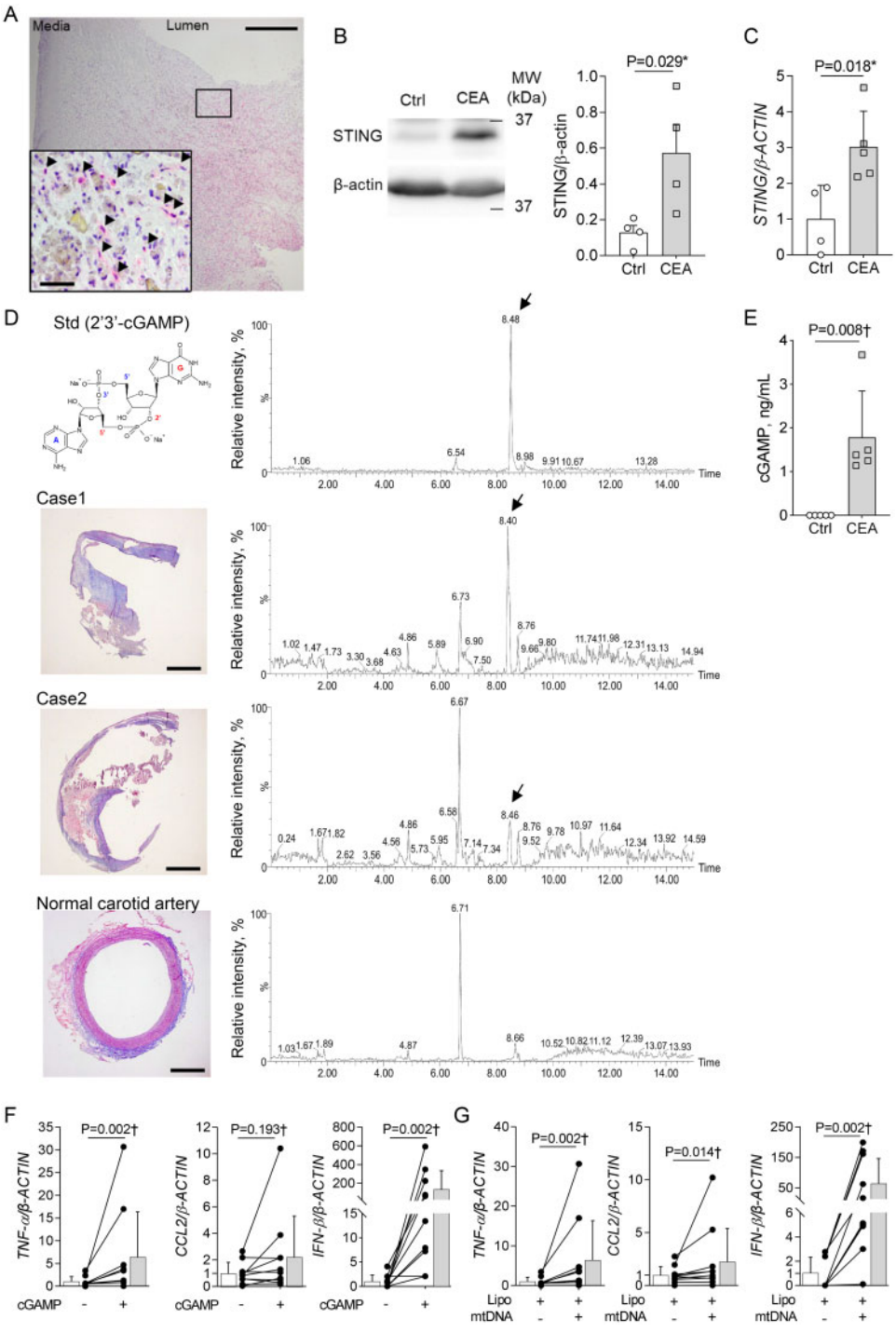


Figure 7 Expression of stimulator of interferon genes and cGAMP in atherosclerotic lesions in human carotid artery. (*A*) Representative image of immunostaining against stimulator of interferon genes in atherosclerotic lesions obtained by carotid endarterectomy. Atherosclerotic lesions in humans expressed stimulator of interferon genes (arrow heads) (n = 4). Scale bar; 500 μm (inset, 50 μm). (*B* and *C*) Western blot analysis (*B*) and qPCR analysis (*C*) indicated that stimulator of interferon genes expression was higher in human carotid artery atherosclerotic lesions obtained by carotid endarterectomy compared with normal carotid arteries (n = 4–5). (*D* and *E*) Representative Masson's trichrome staining and chromatogram of human carotid artery atherosclerotic lesions (n = 5) and normal carotid arteries (n = 4). cGAMP was detected only in human atherosclerotic lesions by LC-MS/MS. The retention time was 8.48 min for standard and 8.40–8.46 min for atherosclerotic lesions. Arrows indicated the peak for cGAMP. cGAMP was quantified using calibration curve, which plotted cGAMP concentration and the peak area. (*F* and *G*) Paired comparison between non-treatment and cGAMP- (*F*) or mtDNA- (*G*) treatment demonstrated that cGAMP or mtDNA promoted the expression of inflammatory molecules in human primary macrophages (n = 10). *Analysed with Student's t-test. †Analysed with Mann–Whitney U test. All values are mean ± SD.

other hand, we could not detect cGAMP in normal carotid arteries ($n = 4$) (*Figure 7D* and *E*). In addition, treatments with cGAMP or mtDNA promoted the expression of inflammatory molecules in human primary macrophages (*Figure 7F* and *G*). These results suggested that STING signalling participates, at least partially, in the development of atherosclerosis in humans.

Discussion

Atherosclerosis is a chronic inflammatory disease[1]; however; understanding the molecular mechanisms of chronic inflammation in the vasculature remains a major medical challenge. The present study addressed a new insight into the underlying mechanism of atherosclerosis associated with STING signalling, which was originally known as a cytosolic DNA-sensing mechanism. Here, we found that atherosclerotic lesions obtained from both human and mouse expressed STING and its ligand, cGAMP. Genetic or pharmacological blockade of STING, or BM-specific expression of STING in *Apoe*$^{-/-}$ mice demonstrated the contribution of STING signalling to the development of atherosclerosis partially through the TBK1 and NF-κB pathways without alteration of metabolic parameters and blood pressure. The results of *in vitro* experiments using human and mouse macrophages indicated the involvement of STING signalling in pro-inflammatory activation of macrophages. These results suggested a pivotal role of STING in atherogenesis (*Graphical abstract*).

The contribution of the innate immune system activated by endogenous ligands to the pathophysiology of various inflammatory diseases including atherosclerosis has attracted much attention.[2,5] Exogenous nucleic acids, especially those from bacteria and viruses, strongly induce inflammation through the recognition by DNA sensors for self-defence,[3] while recent studies have revealed that DNA fragments released from or accumulated in host cells also provoke inflammation as endogenous ligands by using DNA sensors.[34] In addition to TLR9, a most studied DNA sensor, recent studies suggested a link between STING and various disease conditions.[26–32] Furthermore, several studies have demonstrated that inherited activating mutation of STING is associated with vasculopathy in humans,[35–37] suggesting a link between STING signalling and vascular inflammation. Also, other studies reported the contribution of STING to the development of sporadic aortic aneurysm[28] or insulin resistance,[29] both of which, as well as atherosclerosis, share chronic inflammation as an underlying mechanism. However, the role of STING signalling in the development of vascular inflammation and atherosclerosis remains largely unknown.

In this study, we observed features of DNA damage such as the expression of γH2AX and p53, and the accumulation of ssDNA in macrophages in the atherosclerotic aorta obtained from *Apoe*$^{-/-}$ mice, a hypercholesterolaemic mouse model, suggesting release and accumulation of DNA fragments in atherosclerotic lesions, which consequently activate DNA sensors including STING. In fact, the level of cGAMP, a STING agonist, in the aorta was higher in *Apoe*$^{-/-}$ mice, and cGAMP increased inflammatory molecule expression in *Apoe*$^{-/-}$ macrophages but not in *Sting*$^{-/-}$*Apoe*$^{-/-}$ macrophages. Next, we examined whether DNA fragments cause pro-inflammatory activation of macrophages via STING signalling, because cGAMP generated by the action of cGAS from DNA fragments in the cytosol activates STING as a secondary messenger.[38] mtDNA, as expected, markedly promoted inflammatory molecule expression in *Apoe*$^{-/-}$ macrophages compared with *Sting*$^{-/-}$*Apoe*$^{-/-}$ macrophages. The results of immunohistochemistry on atherosclerotic lesions indicated that the expression of anti-inflammatory macrophage marker was associated with the absence of STING, supporting pro-inflammatory role of STING in macrophage activation. The results of western blotting demonstrated that both cGAMP and mtDNA activate the NF-κB and TBK1 pathways, major pathways under STING signalling, in macrophages consistent with the results of our *in vivo* study. Meanwhile, C-176, a STING inhibitor, diminished cGAMP- or mtDNA-induced macrophage activation (e.g. *Tnf-α*, *Ccl2*, and *Ifn-β* expression). On the other hand, a TBK1 inhibitor suppressed the expression of these molecules from macrophage induced by cGAMP; however, in the response to mtDNA, the TBK1 inhibitor suppressed only IFN-β expression. This result suggests that other DNA sensors and/or pathways might also play roles in mtDNA-induced macrophage activation. It is important to note that we revealed the presence of cGAMP in the atherosclerotic aorta by using a highly sensitive LS-MS/MS method. Previous studies proposed an interaction between STING and self-derived DNA and its contribution to disease processes; however, detection of cGAMP was carried out only at the cellular level in *in vitro* experiments.[39,40] Our results demonstrating cGAMP in atherosclerotic lesions in humans and mice provide evidence for the role of STING signalling in the development of atherosclerosis. Our results suggested a pivotal role of STING activation by self-derived DNA in provoking inflammatory activation of macrophages, at least partially, contributing to the development of vascular inflammation and atherosclerosis.

In this study, we also demonstrated that pharmacological blockade of STING using C-176 suppressed atherosclerotic lesion development and lipid deposition in plaques partially through TBK1 inhibition, although its effects were relatively modest. A previous study also demonstrated that inhibition of TBK1 could improve sporadic aortic aneurysm development in different mouse model.[28] Thus, our present study suggested that STING might be a therapeutic target against vascular inflammation and atherogenesis. DNA-sensing mechanisms generally have a protective role in self-defence as part of the innate immune system, although this system also promotes inflammation inappropriately in certain disease contexts. However, the initiating stimuli still remain obscure. Further studies are required to establish therapeutic strategies targeting STING against atherosclerotic vascular diseases.

There are several limitations in this study. First, we used *Apoe*$^{-/-}$ mice fed a WTD. The role of STING was partially confirmed with normal chow-fed *Apoe*$^{-/-}$ mice, although our mouse models induce atherogenesis strongly. In addition, ApoE is a multifunctional protein. Therefore, this model might not be the best model to investigate the role of STING in atherogenesis, especially for humans. Second, we observed the difference in bodyweight between *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$ mice under WTD-fed condition, although there were no phenotypical differences including metabolic parameters between these two strains of mouse. We also could not observe differences in bodyweight in other experiments such as BM transplantation and pharmacological blockade. In addition, plasma triglyceride level was higher in *Sting*$^{-/-}$*Apoe*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice under NC-fed condition, though there was no

difference between bodyweight and other lipid levels. Several studies suggested a link between metabolic parameters and innate immune system.[41–43] Further studies are required to reveal the effects of STING on metabolic parameters. Third, we demonstrated the presence of DNA damage and cGAMP in hypercholesterolaemia-associated atherosclerotic mouse aorta; however, the mechanisms for DNA damage and subsequent release of DNA fragments are not fully understood. Other metabolic abnormalities may also contribute to STING activation through DNA damage. Clarifying the mechanism by which lifestyle-related diseases cause DNA damage requires further studies. Fourth, the number of samples in our clinical study was small, and a large-scale study is required to confirm our results. We used macroscopically normal carotid artery samples as the control, although these might not serve as the adequate control because of the difference in background including surgical methods for sample collection. To examine the role of STING in atherogenesis, further studies targeting other part of arteries such as the coronary artery may be needed because atherosclerotic disease has broad and complex spectrum.[44]

Our study demonstrated that STING signalling contributes to the development of atherogenesis via pro-inflammatory activation of macrophages. We also demonstrated the presence of STING and cGAMP in human atherosclerotic lesions, providing clinically translatable evidence for the pro-inflammatory and pro-atherogenic role of STING signalling. Stimulator of interferon genes signalling may explain the link between innate immunity and chronic inflammation caused by lifestyle-related diseases. In conclusion, DNA damage caused by lifestyle-associated diseases and subsequent STING activation plays, at least partially, a causal role in atherosclerosis. Our findings shed new insights into the pathogenesis of vascular inflammation and atherogenesis, and suggest that STING signalling might be a potential therapeutic target against atherosclerotic vascular diseases.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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