STAT3/NF-κB decoy oligodeoxynucleotides inhibit atherosclerosis through regulation of the STAT/NF-κB signaling pathway in a mouse model of atherosclerosis

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Abstract. Atherosclerosis is a progressive chronic inflammatory condition that is the cause of most cardiovascular and cerebrovascular diseases. The transcription factor nuclear factor-κB (NF-κB) regulates a number of genes involved in the inflammatory responses of cells that are critical to atherogenesis, and signal transducer and activator of transcription (STAT)3 is a key transcription factor in immunity and inflammation. Decoy oligodeoxynucleotides (ODNs) bind to sequence-specific transcription factors and limit gene expression by interfering with transcription *in vitro* and *in vivo*. The present study aimed to investigate the beneficial functions of STAT3/NF-κB decoy ODNs in liposaccharide (LPS)-induced atherosclerosis in mice. Atherosclerotic injuries of mice were induced via intraperitoneal injection of LPS and the mice were fed an atherogenic diet. Ring-type STAT3/NF-κB decoy ODNs were designed and administered via an injection into the tail vein of the mice. To investigate the effect of STAT3/NF-κB decoy ODNs, electrophoretic mobility shift assay, western blot analysis, histological analysis with hematoxylin and eosin staining, Verhoeff-Van Gieson and Masson's trichrome staining were performed. The results revealed that STAT3/NF-κB decoy ODNs were able to suppress the development of atherosclerosis by attenuating morphological changes and inflammation in atherosclerotic mice aortae, and by reducing pro-inflammatory cytokine secretion through inhibition of the STAT3/NF-κB pathway. In conclusion, the present study provided novel insights into the antiatherogenic molecular mechanism of STAT3/NF-κB decoy ODNs, which may serve as an additional therapeutic intervention to combat atherosclerosis.

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Introduction

Atherosclerosis is a chronic degenerative arterial condition, and is a leading cause of most cerebrovascular and cardiovascular diseases via its complex and progressive effects on the arterial wall (1,2). Atherosclerosis is characterized by the abnormal accumulation of fibrous components and lipids in the intima, resulting in arterial wall thickening and a reduction in the size of the vascular cavity (1,3). Vascular inflammation in atherosclerosis is accompanied by an accumulation of cholesterol, lipids, calcium and cellular debris within the vessel wall intima (4). This deposition can result in plaque production, revascularization, acute and chronic lumen obstruction, blood flow abnormalities and reduced oxygen supply to the target organs (5). The mechanisms underlying the pathology of atherosclerosis are complex, but mainly consist of endothelial cell dysfunction, macrophage polarization, inflammation and the immune response (1).

Inflammation is involved in all stages of atherosclerosis, ranging from endothelial cell injury to the ultimate rupture of plaques (3). Furthermore, it has been reported that chronic inflammation may be an independent risk factor for atherosclerosis (6). Critical factors that can contribute to the early stages of atherosclerosis and plaque development are pro-inflammatory cytokines, including interleukin-6 (IL-6), interferon (IFN)-α, IFN-γ and Toll-like receptor 4 (TLR4) (7). Notably, nuclear factor-κB (NF-κB) activation is required for the regulation of a number of genes involved in the inflammatory response of cells that are critical to atherogenesis. The activation of NF-κB results in the subsequent transcription of pro-inflammatory genes, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), IL-1β and TNF-α; therefore, pro-inflammatory genes are principally regulated by NF-κB (8).

The signal transducer and activator of transcription (STAT) family of transcription factors can activate key mediators of cytokine responses; within this family, STAT3 is a principal transcription factor associated with immunity and inflammation (9). Notably, STAT3 has a crucial regulatory role in cell survival and proliferation; its activation has been detected in numerous human tumors, including melanoma, head and neck squamous cell carcinoma, multiple myeloma, mantle cell lymphoma, glioma, and colon, lung, breast, pancreas and prostate cancer (10,11). In addition, STAT3 has been reported to be associated with various cardiovascular diseases, including arteriosclerosis, cardiac hypertrophy and heart failure (6). STAT3 may serve a crucial role in all of these diseases through endothelial cell dysfunction, macrophage polarization, inflammation and the immune response, thus indicating that STAT3 may be a potential new target of atherosclerosis therapies (1). Therefore, inhibiting NF-κB and STAT3 expression may prevent the formation of atherosclerosis and slow its progression. Therefore, the present study attempted to suppress atherosclerosis by blocking the NF-κB and STAT3 transcription factors to regulate inflammation.

Decoy oligodeoxynucleotides (ODNs) inhibit target gene expression through the consensus binding-site sequences of target transcription factors (12). Consequently, decoy ODNs bind to sequence-specific transcription factors and limit gene expression by interfering with transcription *in vitro* and *in vivo* (13). Transfection of cis-element double-stranded ODNs attenuates authentic cis-trans interactions, resulting in the removal of trans factors from endogenous cis elements and the subsequent modulation of gene expression (13). Gene therapy based on decoy ODNs may be useful for treating a number of diseases, including glomerulonephritis, myocardial infarction and rheumatoid arthritis, and may prevent acute rejection after renal transplantation (14). Our previous study demonstrated the anti-atherosclerotic effects of NF-κB decoy ODNs on pro-inflammatory cytokines and adhesion molecules in a mouse model of lipopolysaccharide (LPS)-induced atherosclerosis (15). Furthermore, our previous study demonstrated the effect of SREBP decoy ODNs on a mouse model of non-alcoholic fatty liver disease in high-fat diet-induced hyperlipidemia (12). However, to the best of our knowledge, the effect of STAT3/NF-κB decoy ODNs on an animal model of atherosclerosis has not yet been investigated.

The present study hypothesized that STAT3/NF-κB decoy ODNs may suppress the development of atherosclerosis in a mouse model by simultaneously inhibiting the STAT/NF-κB signaling pathway and its related inflammatory reaction. The aim of this study was to determine the useful functions and possible underlying molecular mechanisms of STAT3/NF-κB decoy ODNs in a mouse model of bacterial endotoxin LPS-induced atherosclerosis.

Materials and methods

Construction and synthesis of decoy ODNs. Synthetic decoy ODNs were synthesized by Macrogen, Inc. using the following ODN sequences (the target sites of the consensus-binding sequences are underlined): STAT3 decoy ODN, 5'-GAATTCCTTCTGGGAATTCCAAAAGGAATTCCCAGAAG-3'; NF-κB decoy ODN, 5'-GAATTCAGGGAAATCCCTTCAAGAAAACTTGAAGGGATTTCCCT-3'; and scramble (Scr) ODN, 5'-GAATTCAATTCAGGGTACGGCAAAAAATTGCCGTACCCTGAATT-3'. Subsequently, STAT3, NF-κB, STAT3/NF-κB decoy ODNs and Scr decoy ODN were annealed for 6 h, decreasing the temperature from 80 to 25˚C during this time. To obtain a covalent ligation for ring-type decoy ODNs, each ODN was mixed with T4 DNA ligase (Takara Bio, Inc.) and incubated for 18 h at 16˚C. These decoy ODNs were predicted to form a covalently ligated ring-type structure (Fig. 1A).

Atherosclerosis model. A total of 60, male C57BL/6 mice (age, 6 weeks; weight, 20-25 g; Samtaco Bio Korea Co., Ltd.) were housed in a room at a controlled temperature of 22±2˚C and a humidity of 55%, under a 12-h light/dark cycle. The mice were given free access to water and food. Atherosclerotic injuries were induced via intraperitoneal injection of LPS from *Escherichia coli* O111:B4 (2 mg/kg body weight; dissolved in 200 μl PBS; MilliporeSigma) once a week for 8 weeks. Simultaneously, the mice were fed an atherogenic diet (AD; 21.2% milkfat, 1.25% cholesterol, 0.5% cholic acid; DooYeol Biotech.). Decoy ODNs (10 μg) were administered every 2 weeks for 8 weeks via an injection into the tail vein using a Trans IT *in vivo* gene delivery system (Mirus Bio.). The mice were divided into the following six groups (n=10 mice/group): i) Untreated group [normal control (NC)], ii) STAT3/NF-κB decoy ODN-treated group (STAT/NF-κB) fed a standard diet (cat. no. 2018S; Envigo), iii) Scr decoy ODN-treated group injected with LPS and fed an AD (LPS + AD + Scr), iv) STAT3 decoy ODN-treated group injected with LPS and fed an AD (LPS + AD + STAT), v) NF-κB decoy ODN-treated group injected with LPS and fed an AD (LPS + AD + NF-κB), vi) STAT3/NF-κB decoy ODN-treated group injected with LPS and fed an AD (LPS + AD + STAT/NF-κB). All mice were anesthetized with 2-3% isoflurane inhalation (Ifran; HANA Pharm Co., Ltd.) using an RC2 Rodent Circuit Controller (VetEquip, Inc.) before tail vein injection. At the end of each treatment period, blood was collected by cardiac puncture from the mice, and the mice were euthanized by asphyxiation with 60-70% CO_2 of the cage volume/min. Subsequently, their aorta and heart tissues were excised for subsequent experiments. The humane endpoints in the present study were as follows: i) The animals showed hypothermia (<37˚C) in the absence of anesthesia; ii) the animal lost 20% of its original weight; iii) the animal exhibited loss of ability to ambulate (unable to access food or water). All animals reaching these endpoints were euthanized with 60-70% CO_2 of the cage volume/min. Death verification included the absence of heartbeat, breathing or respiration. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (Daegu, South Korea; approval no. DC IAFCR-181204-27Y). All procedures performed in experiments involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Electrophoretic mobility shift assay (EMSA). Nuclear extract fractionation was performed on the abdominal aorta tissues of mice using an NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Inc.) according to

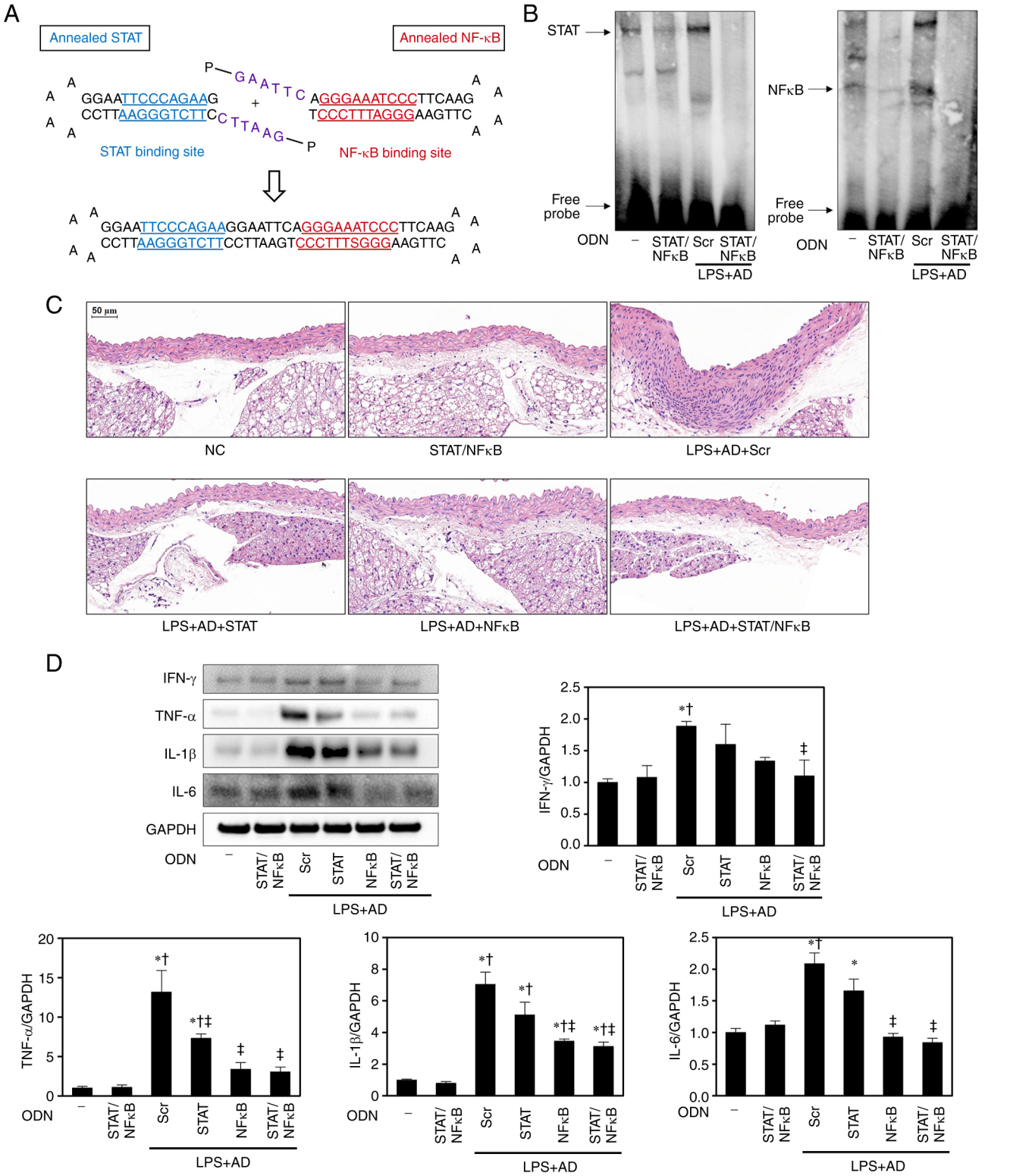

Figure 1. STAT/NF-κB decoy ODNs suppress histological changes and inflammation in atherosclerotic mice aortae. (A) Structure of STAT/NF-κB synthetic decoy ODNs. (B) Electrophoretic mobility shift assay was performed to analyze the effects of STAT/NF-κB synthetic decoy ODNs on STAT and NF-κB-binding activity in atherosclerotic mice (n=5). (C) Histopathological alterations were determined by hematoxylin and eosin staining; representative images from each group are shown (n=5). Scale bar, 50 μm. (D) Western blotting was performed to detect the expression levels of inflammatory cytokines in aorta tissues. The graph summarizes the semi-quantification of protein expression normalized to GAPDH (n=3). *P<0.05 vs. NC group; †P<0.05 vs. STAT/NF-κB group; ‡P<0.05 vs. LPS + AD + Scr group. AD, atherogenic diet; IFN-γ, interferon-γ; IL, interleukin; NC, normal control; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; LPS, lipopolysaccharide; Scr, scramble; STAT, signal transducer and activator of transcription.

the manufacturer's instructions based on standard protocols. Subsequently, a Lightshift Chemiluminescent EMSA kit (cat. no. 20148; Thermo Fisher Scientific, Inc.) was performed to analyze STAT3 and NF-κB DNA binding activation. ODNs containing the consensus STAT3 and NF-κB binding sites (STAT3, forward 5'-GATCCTTCTGGGAATTCCTAG

ATC-3', reverse 5'-GATCTAGGAATTCCCAGAAGGATC-3'; NF-κB, forward 5'-CTTGAAGGGATTTCCCTGGCT-3', reverse 5'-AGCCAGGGAAATCCCTTCAAG-3') and 3' end-labeled with biotin were used as probes. Biotin-labeled probes were synthesized by Macrogen, Inc. The probes (20 fmol) were subjected to a denaturation step at 90˚C for 1 min followed by annealing at room temperature for 30 min. Then, for the binding reaction, the binding reaction components were added in the order listed according to the manufacturer's instructions. At this time, 10 μg nuclear extract was added to binding reactions and incubated for 20 min at room temperature. The nuclear protein concentration (1-10 μg/μl) was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc.). The Novex™ TBE gels 4-12% (cat. no. EC62352; Thermo Fisher Scientific, Inc.) were used for pre-electrophoresis for 30-60 min at 100 V. The sample complexes were separated by electrophoresis on gels using 0.5X TBE (cat. no. LC6675; Thermo Fisher Scientific, Inc.) as a running buffer until the bromophenol blue dye has migrated ~3/4 down the length of the gel. After electrophoresis, the gels were transferred to nylon membranes and detected using the Streptavidin-Horseradish Peroxidase Conjugate and the Chemiluminescent Substrate (cat. no. 89880; Thermo Fisher Scientific, Inc.), according to manufacturer's instructions. The signal intensity was detected using an image analyzer (ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc.).

Atherosclerosis lesion analyses. All abdominal aorta and heart tissue specimens were fixed in 10% formalin for 24 h at room temperature. Thereafter, sections of the aorta and heart were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. The sections (4 μm) were mounted on glass slides, rehydrated in distilled water and stained with hematoxylin and eosin (H&E), Verhoeff-Van Gieson and Masson's trichrome based on standard protocols. The tissue sections were deparaffinized and stained with hematoxylin at room temperature for 8 min and then with eosin at room temperature for 5 min. For the Verhoeff-Van Gieson stain, the tissue sections were deparaffinized and stained with alcoholic hematoxylin, 10% ferric chloride and Weigert's iodine mixed solution for 5 min at room temperature. After differentiating in 2% ferric chloride for 1-2 min and treating with sodium thiosulfate for 1 min, the sections were counterstained with Van Gieson's solution for 40 sec. For the Masson's trichrome stain, the tissue sections were deparaffinized and refixed in Bouin's solution for 30 min at 60˚C. After being stained at room temperature with Weigert's hematoxylin and a Biebrich scarlet-acid fuchsin each for 10 min, the sections were treated in phosphomolybdic–phosphotungstic acid for 10 min and finally stained with aniline blue for 10 min. All slides were examined under a Pannoramic MIDI slide scanner (3DHISTECH Kft.).

Western blot analysis. The aorta tissue specimens were lysed in protein lysis buffer (CelLytic™ M; MilliporeSigma) for 20 min on ice and were then centrifuged at 13,800 x g for 20 min at 4˚C. Nuclear and cytosolic protein samples were prepared from the aorta tissue using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The supernatant was collected and the protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Inc.). The protein samples were then loaded on precast gradient SDS polyacrylamide gels (Bolt™ 4-12% Bis-Tris Plus Gels; Thermo Fisher Scientific, Inc.) and transferred to nitrocellulose membranes (GE Healthcare) using a Bolt™ Mini Blot Module and Mini Gel Tank (Thermo Fisher Scientific, Inc.), according to the manufacturer's recommendations. The membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (MilliporeSigma) and incubated overnight with primary antibodies at 4˚C. After primary antibody incubation, horseradish peroxidase (HRP)-conjugated secondary antibodies were used to incubate the membranes for 2 h at room temperature. The primary antibodies used in the present study were as follows: Anti-IFN-γ (1:1,000; cat. no. ab9657), anti-TNF-α (1:1,000; cat. no. ab1793), anti-IL-6 (1:1,000; cat. no. ab208113), anti-ATP-binding cassette transporter A1 (ABCA1; 1:500; cat. no. ab18180), anti-monocyte chemoattractant protein-1 (MCP-1; 1:1,000; cat. no. ab21396), anti-fibronectin (1:1,000; cat. no. ab2413), anti-α-smooth muscle actin (α-SMA; 1:1,000; cat. no. ab5694) (all from Abcam), anti-IL-1β (1:1,000; cat. no. sc-32294), anti-COL1A2 (1:500; cat. no. sc-8788), anti-TLR4 (1:1,000; cat. no. sc-10741), anti-JAK2 (1:1,000; cat. no. sc-294) (all from Santa Cruz Biotechnology, Inc.), anti-ICAM-1 (1:1,000; cat. no. AF796), anti-VCAM-1 (1:1,000; cat. no. AF643) (both from R&D Systems, Inc.), anti-phosphorylated (P)-JAK2 (1:1,000; cat. no. 8082), anti-P-IκB (1:1,000; cat. no. 2859), anti-IκB (1:1,000; cat. no. 9242), anti-NF-κB (1:1,000; cat. no. 8242), anti-P-NF-κB (1:1,000; cat. no. 3033), anti-STAT3 (1:1,000; cat. no. 9139), anti-P-STAT3 (1:1,000; cat. no. 9145), anti-GAPDH (1:2,000; cat. no. 2118) (all from Cell Signaling Technology, Inc.) and anti-Lamin B1 (1:1,000; cat. no. 332000; Invitrogen; Thermo Fisher Scientific, Inc.). The secondary antibodies used in the present study were as follows: Anti-mouse (1:1,000; cat. no. 7076) and anti-rabbit (1:1,000; cat. no. 7074) (both from Cell Signaling Technology, Inc.). After washing, the membranes were visualized using enhanced chemiluminescence detection reagents (SuperSignal™ West Femto Maximum Sensitivity Substrate; cat. no. 34096; Thermo Fisher Scientific, Inc.) for 1 min. The signal intensity was detected using an image analyzer (ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc.) and was semi-quantified with Image Lab software version 5.1 (Bio-Rad Laboratories, Inc.).

Immunohistochemical (IHC) staining. Paraffin-embedded tissue sections (4 μm) were placed in a BOND-MAX Fully Automated IHC Staining System slide stainer (Leica Microsystems, Inc.) according to the following protocol. First, tissues were deparaffinized and pre-treated with the Epitope Retrieval Solution 2 (EDTA-buffer pH 8.8) at 98˚C for 20 min. After washing steps, peroxidase blocking was carried out for 10 min using the BOND Polymer Refine Detection Kit (cat. no. DS9800; Leica Microsystems, Inc.). Tissues were washed again and then incubated with the primary antibodies (1:100) for 30 min at room temperature. The following primary antibodies were used: Anti-ICAM-1, anti-VCAM-1 (both from R&D Systems) and anti-monocyte + macrophage (MOMA-2; cat. no. ab33451; Abcam). The tissue sections were then incubated with post-primary for 8 min and were then incubated with polymer for 8 min and developed with DAB-Chromogen

for 10 min at room temperature. Hematoxylin was used as the counterstain for 5 min at room temperature. The slides were examined using a Pannoramic MIDI slide scanner.

Statistical analysis. All data are presented as the mean ± standard error of the mean. Statistical significance was assessed using one-way analysis of variance with Tukey's multiple comparison test using GraphPad Prism 5.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Construction of decoy ODNs. The present study first designed STAT3, NF-κB and STAT3/NF-κB decoy ODNs (Fig. 1A). Decoy ODNs containing the DNA-binding consensus sequences of transcription factors selectively inhibit STAT3 and NF-κB by binding to the DNA-binding domains. To identify the functions of STAT3/NF-κB decoy ODNs in controlling STAT3 and NF-κB expression, EMSA was performed with nuclear extracts obtained from the aorta tissues of mice with LPS/AD-induced atherosclerosis and treated with Scr and STAT3/NF-κB decoy ODNs. Compared with in NC mice, LPS + AD + Scr atherosclerotic mice exhibited increased STAT3 and NF-κB DNA-binding activity. However, the LPS + AD + STAT/NF-κB treatment groups exhibited reduced STAT3 and NF-κB DNA-binding activity compared with the LPS + AD + Scr group (Fig. 1B). These results indicated that STAT3/NF-κB decoy ODNs effectively decreased STAT and NF-κB expression.

STAT3/NF-κB decoy ODNs attenuate morphological changes and inflammation in atherosclerotic mice aortae. To investigate the effect of STAT3/NF-κB decoy ODNs on atherosclerotic mice, H&E histological analysis was performed. A marked increase in the thickness of the adventitia and media, and noticeable cellular infiltration in the adventitial layers of the aortae were detected in the LPS + AD + Scr group compared with in the NC group. Histological analysis showed that these histological changes were markedly decreased in the LPS + AD + STAT/NF-κB groups (Fig. 1C). Furthermore, no differences in body weight were determined among the groups (data not shown).

To examine the mechanism of action of STAT3/NF-κB decoy ODNs, the protein expression levels of IFN-γ, TNF-α, IL-1β and IL-6 were measured in the aorta tissues of atherosclerotic mice using western blot analysis (Fig. 1D). Notably, the expression levels of IFN-γ, TNF-α, IL-1β and IL-6 were significantly higher in the LPS + AD + Scr group than those in the NC and STAT/NF-κB groups. However, in the LPS + AD + STAT/NF-κB group, the increase in these protein expression levels was returned to a near-NC level. These observations suggested that STAT3/NF-κB decoy ODNs can inhibit atherosclerotic morphological changes and inflammation.

STAT3/NF-κB decoy ODNs diminish structural damage in the aortae of atherosclerotic mice. The tissue sections were subjected to histological analysis with Verhoeff-Van Gieson and Masson trichrome staining in order to characterize the abdominal aortic lesions. Besides cellular components, the extracellular matrix (ECM) of atherosclerotic plaque serves a relevant role in the initiation and subsequent progression of atherosclerosis. Major ECM structural and signaling components include elastin and collagen (16). Verhoeff-Van Gieson staining of elastin revealed no elastin fiber fragmentation in the NC and STAT/NF-κB groups. The elastin content in the medial layer was diminished and fragmented in the LPS + AD + Scr atherosclerosis group, perhaps because of the increased medial area or thinning of the elastin fibers; however, the elastin fibers were less disrupted in the LPS + AD + STAT/NF-κB mice compared with that in the LPS + AD + Scr mice (Fig. 2A). In addition, based on Masson's trichrome staining, a marked increase in collagen deposition in the aortae of LPS + AD + Scr group mice was detected, whereas this was markedly reduced in LPS + AD + STAT/NF-κB mice (Fig. 2C). Western blot analysis confirmed these results, showing that the protein expression levels of COL1A2 were increased in the LPS + AD + Scr group compared with those in the NC group, but were significantly decreased following treatment with STAT3/NF-κB decoy ODNs (Fig. 2B). Elastin is one of the dominant ECM proteins and collagen accumulation is characteristic of atherosclerotic plaques (16,17). These results suggested that increased ECM components in the LPS + AD + Scr mice may be attenuated by STAT3/NF-κB decoy ODNs.

STAT3/NF-κB decoy ODNs inhibit morphological changes in the hearts of atherosclerotic mice and regulate cholesterol metabolism. To further investigate the cardioprotective effects of STAT3/NF-κB decoy ODNs, their effects on heart morphology were assessed. H&E staining of heart tissues revealed that myocardial fibers in the NC group were normal and ordered. There were no broken fibers and the nuclei of the myocardial cells were regular. In addition, no marked changes were observed between the NC and STAT/NF-κB groups. The LPS + AD + Scr mice displayed structural abnormalities, with disordered, extensively collapsed and degenerated muscle fibers; however, these histological changes were markedly alleviated by treatment with STAT3/NF-κB decoy ODNs (Fig. 3A).

ABCA1 serves a central role in the early stages of the reverse cholesterol transport pathway by mediating lipid efflux from macrophages and is important for maintaining cellular cholesterol homeostasis (18,19). Therefore, the present study explored the effects and potential mechanisms of STAT3/NF-κB decoy ODNs on ABCA1 expression in atherosclerotic mice. As shown in Fig. 3B, STAT3, NF-κB and STAT3/NF-κB decoy ODNs significantly increased the protein expression levels of ABCA1 compared with those in the LPS + AD + Scr group. Given that ABCA1 expression can be suppressed by pro-inflammatory stimuli via the NF-κB signaling pathway, STAT3/NF-κB decoy ODNs may regulate ABCA1 expression and cholesterol metabolism.

Effects of STAT3/NF-κB decoy ODNs on the expression levels of adhesion molecules in atherosclerotic mice aortae. The expression of ICAM-1 and VCAM-1 in endothelial cells is the earliest known event in the initiation and progression of atherosclerosis (20). To identify the function of STAT3/NF-κB decoy ODNs in ICAM-1 and VCAM-1 expression, IHC

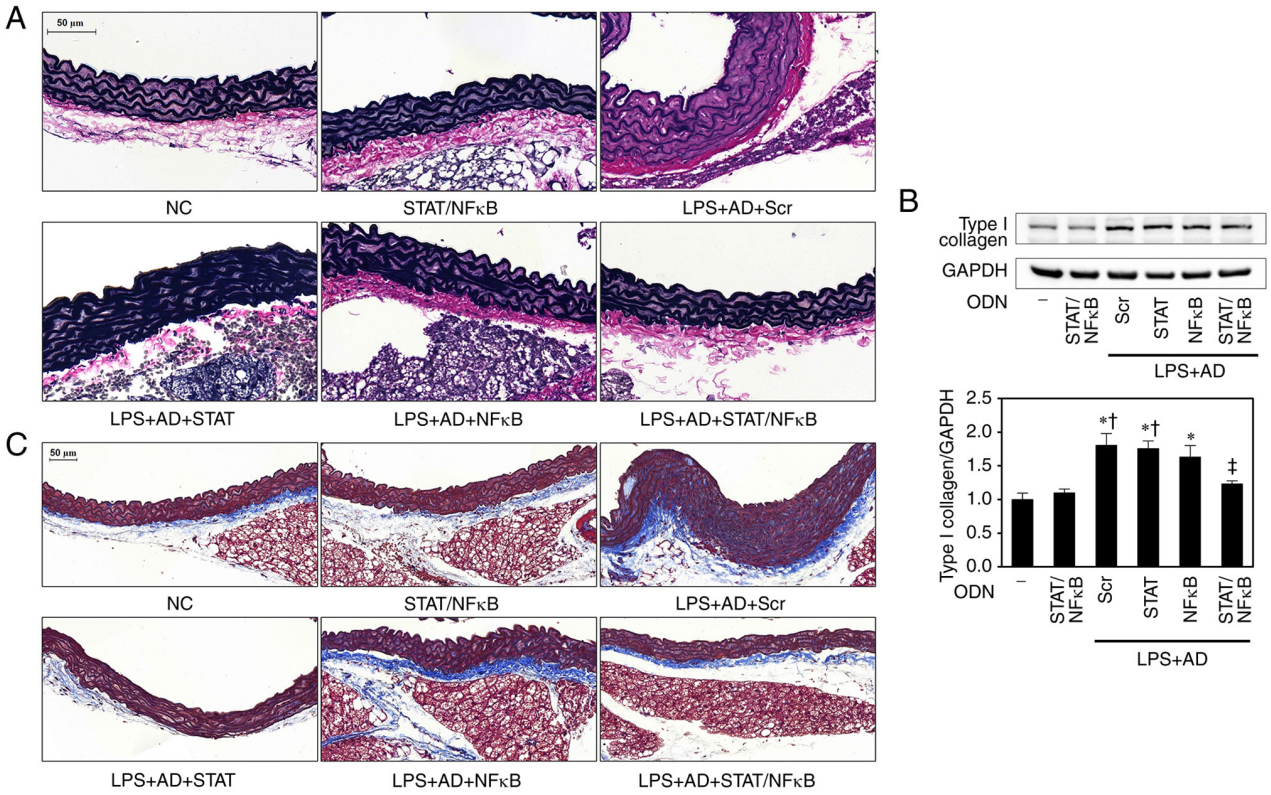

Figure 2. STAT/NF-κB decoy ODNs diminish structural damage in the aortae of atherosclerotic mice. Histopathological alterations in slides stained with (A) Verhoeff-Van Gieson. Representative images from each group are shown (n=5). Scale bar, 50 μm. (B) Western blotting was performed to detect the expression levels of collagen in aorta tissues. The graph summarizes the semi-quantification of molecules of protein expression normalized to GAPDH (n=3). *P<0.05 vs. NC group; †P<0.05 vs. STAT/NF-κB group; ‡P<0.05 vs. LPS + AD + Scr group. (C) Histopathological alterations in slides stained with Masson's trichrome. Representative images from each group are shown (n=5). Scale bar, 50 μm. AD, atherogenic diet; NC, normal control; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; LPS, lipopolysaccharide; Scr, scramble; STAT, signal transducer and activator of transcription.

staining was performed. IHC staining demonstrated that ICAM-1 and VCAM-1 were expressed in some endothelial cells over lesions and in the endothelia adjacent to the lesions in the LPS + AD + Scr mice, whereas the changes in ICAM-1 and VCAM-1 expression were markedly decreased in the LPS + AD + STAT/NF-κB mice (Fig. 4A and C). Western blot analysis further confirmed that ICAM-1 and VCAM-1 expression levels in the aortae of LPS + AD + Scr atherosclerotic mice were higher than those in the NC and STAT/NF-κB groups, whereas these expression levels were decreased in the LPS + AD + STAT/NF-κB mice (Fig. 4B).

Effects of STAT3/NF-κB decoy ODNs on the expression of fibrosis-related proteins in atherosclerotic mice. To determine whether STAT3/NF-κB decoy ODNs may act on fibrosis, the expression levels of fibrosis-related proteins were detected in aortic lesions. Vascular fibrosis involves the excess accumulation of ECM proteins, such as collagen, proteoglycan and fibronectin, in the arterial wall, leading to decreased luminal diameter and increased vascular stiffness, which are characteristics of atherosclerosis (21). Western blot analysis detected the expression levels of the ECM-related marker fibronectin and the contractile smooth muscle cell (SMC)-related marker α-SMA. In the LPS + AD + Scr group the aortic protein expression levels of fibronectin and α-SMA were significantly increased compared with those in the NC and STAT/NF-κB groups. By contrast, the expression levels of fibronectin and α-SMA were significantly decreased in the LPS + AD + STAT/NF-κB mice compared with those in the LPS + AD + Scr group (Fig. 5A). MCP-1 serves a crucial role in initiating atherosclerosis by recruiting macrophages and monocytes to the vessel walls (22). The results of the present study showed that the expression levels of MCP-1 were increased in the LPS + AD + Scr group compared with those in the NC and STAT/NF-κB groups. Conversely, the expression levels of MCP-1 were inhibited in the LPS + AD + STAT/NF-κB group (Fig. 5A).

IHC staining was also performed with the common macrophage marker MOMA-2 (Fig. 5B). The progressive deposition of inflammatory cells, such as macrophages, promotes plaque vulnerability leading to enlarged unstable plaques covered by a thin fibrous cap (23). MOMA-2-positive cells were not detected in the NC and STAT/NF-κB groups, but were observed in the LPS + AD + Scr group. Moreover, macrophage accumulation was markedly reduced in the LPS + AD + STAT/NF-κB atherosclerotic lesions.

STAT3/NF-κB decoy ODNs inhibit activation of the JAK/STAT and TLR4/NF-κB pathways in LPS-induced inflammation. Numerous inflammatory signaling pathways that contribute to the pathogenesis of atherosclerosis are modulated by the transcription factor NF-κB, which is a master regulator of innate and adaptive immune responses (24). In addition, TLR4 serves a crucial role in the initiation of an innate immune

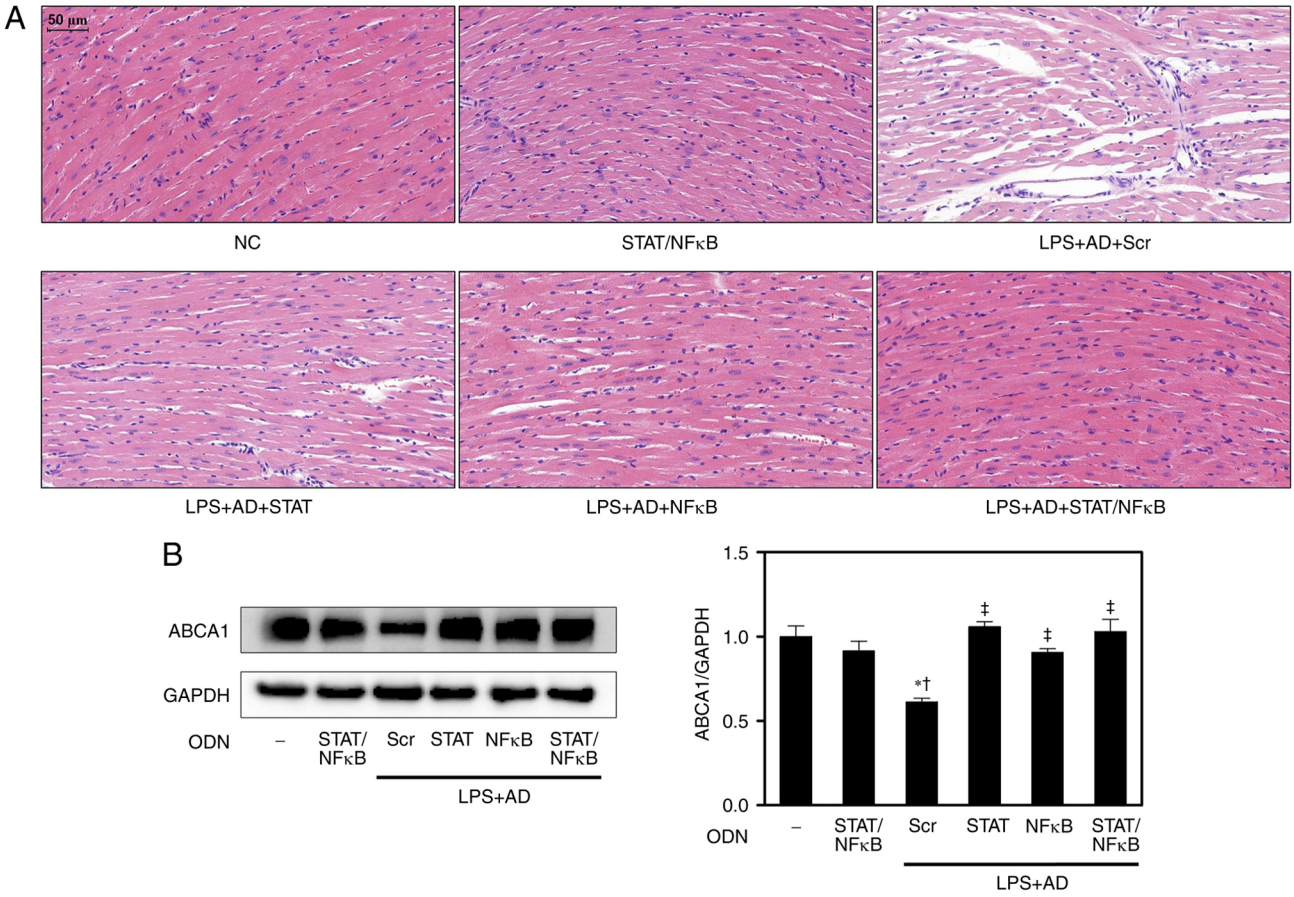

Figure 3. STAT/NF-κB decoy ODNs attenuate morphological change in atherosclerotic mouse hearts and improve cholesterol metabolism. (A) Histological images of hematoxylin and eosin staining of atherogenic mouse hearts. Representative images from each group are shown (n=5). Scale bar, 50 μm. (B) Western blotting was performed to detect the expression levels of ABCA1 expression in aorta tissues. The graph summarizes the semi-quantification of molecules of protein expression normalized to GAPDH (n=3). *P<0.05 vs. NC group; †P<0.05 vs. STAT/NF-κB group; ‡P<0.05 vs. LPS + AD + Scr group. ABCA1, ATP-binding cassette transporter A1; AD, atherogenic diet; NC, normal control; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; LPS, lipopolysaccharide; Scr, scramble; STAT, signal transducer and activator of transcription.

response through activation of inflammatory cells via the NF-κB-dependent pathway (24). Therefore, the present study examined the effects of STAT3/NF-κB decoy ODNs on LPS-stimulated inflammation via the TLR4/NF-κB signaling pathway in atherosclerotic mice. Western blot analysis indicated that the protein expression levels of TLR4 were increased in the LPS + AD + Scr group compared with those in the NC group; however, were decreased following treatment with STAT3/NF-κB decoy ODNs (Fig. 5C). Because the activity of NF-κB is regulated by IκBα, the effect of STAT3/NF-κB decoy ODNs on the phosphorylation of IκBα was assessed. As shown in Fig. 5C, in a mouse model of atherosclerosis, the expression levels of P-IκBα were increased in the cytoplasm and the expression levels of P-NF-κB were increased in the nucleus. In the LPS + AD + STAT/NF-κB mice it was indicated that that STAT3/NF-κB decoy ODNs may inhibit NF-κB activation by inhibiting P-IκBα. These results suggested that STAT3/NF-κB decoy ODNs may markedly inhibit the LPS-activated TLR4/NF-κB signaling pathway in a mouse model of atherosclerosis.

The JAK/STAT signaling pathway has a vital role in various important cellular responses, such as inflammation, metabolism, cell proliferation and gene transcription (25). Western blot analysis was performed to detect the protein expression levels of JAK2 and STAT3 in the aortae. As shown in Fig. 5C, the LPS + AD + Scr group exhibited markedly enhanced P-JAK2 and P-STAT3 expression compared with that in the NC group. By contrast, the LPS + AD + STAT/NF-κB groups exhibited decreased P-JAK2 and P-STAT3 expression levels compared with those in the LPS + AD + Scr group. Taken together, these findings suggested that STAT3/NF-κB decoy ODNs may suppress inflammation in atherosclerotic mice by effectively inhibiting the STAT/NF-κB signaling pathway.

Discussion

The present study explored the anti-atherosclerotic effects and molecular mechanisms of STAT3/NF-κB decoy ODNs in LPS-induced atherosclerotic mice. The results revealed that STAT3/NF-κB decoy ODNs can attenuate the development of atherosclerosis by promoting ABCA1-mediated cholesterol efflux, and reducing pro-inflammatory cytokine secretion and fibrosis-related protein expression via suppression of the STAT/NF-κB pathway.

Atherosclerosis is characterized by the progressive formation of vascular lesions caused by immoderate lipid deposition and a chronic inflammatory response within the arterial walls, subsequently leading to ischemic

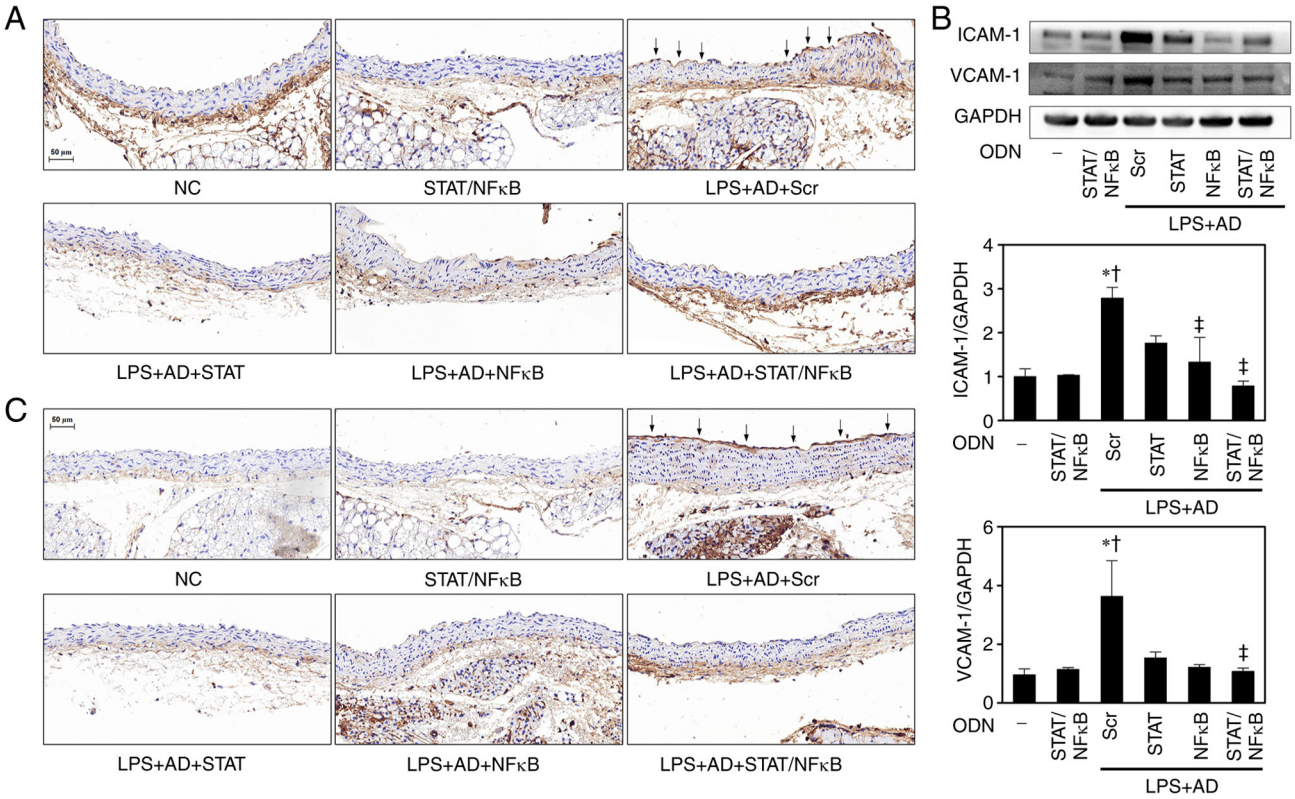

Figure 4. STAT/NF-κB decoy ODNs alleviate atherosclerotic mice aortae injury. Immunohistochemistry images of (A) ICAM-1. Representative images from each group are shown (n=5). Arrows indicate areas of respective adhesion molecule expression. Scale bar, 50 μm. (B) Western blotting was performed to detect the protein expression levels of adhesion molecules in aorta tissues. The graph summarizes the semi-quantification of molecules of protein expression normalized to GAPDH (n=3). *P<0.05 vs. NC group; †P<0.05 vs. STAT/NF-κB group; ‡P<0.05 vs. LPS + AD + Scr group. (C) Immunohistochemistry images of VCAM-1. Representative images from each group are shown (n=5). Arrows indicate areas of respective adhesion molecule expression. Scale bar, 50 μm. AD, atherogenic diet; ICAM-1, intercellular adhesion molecule-1; NC, normal control; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; LPS, lipopolysaccharide; Scr, scramble; STAT, signal transducer and activator of transcription; VCAM-1, vascular cell adhesion molecule-1.

cardiovascular and cerebrovascular diseases (26). ABCA1 can regulate foam cell formation via the export of excessive cholesterol from lipid-loaded macrophages, and maintains cellular lipid and cholesterol homeostasis (27). Although its key role is to maintain lipid homeostasis by controlling cellular cholesterol and phospholipid efflux, ABCA1 has gradually been recognized as having anti-inflammatory functions in various diseases in which inflammation is an underlying pathogenic mechanism (28). ABCA1 expression in can promote the export of cellular cholesterol to the extracellular acceptor protein apolipoprotein-A1 (29). ABCA1 expression has been reported to be decreased in atherosclerosis, which is associated with an aggravated intracellular cholesterol accumulation, and enhanced foam cell generation and formation (30). In the present study, ABCA1 expression was significantly decreased in the arteriosclerotic mice, whereas it was significantly increased by treatment with STAT3/NF-κB decoy ODNs.

Atherosclerosis is a chronic inflammatory condition that is characterized by the accumulation of lipids, SMC proliferation, cell apoptosis of SMCs, T lymphocytes and macrophages, necrosis, fibrosis and local inflammation (31,32). The LPS from gram-negative bacteria used in the present study is a natural ligand of TLR4 that stimulates macrophage signaling via MyD88-dependent TLR signaling (8,33). TLR4 stimulation activates the NF-κB transcription factor and pro-inflammatory proteins, which promote inflammation and initiate atherogenesis, as well as the destabilization of atherosclerotic plaques (34). The transcription factor NF-κB directly targets inflammation by increasing the formation of inflammatory cytokines, chemokines and adhesion molecules, and it can also regulate cell proliferation, differentiation, apoptosis and morphogenesis (24). The present study revealed that STAT3/NF-κB decoy ODNs not only inhibited the NF-κB signaling pathway but also regulated TLR4 expression. Furthermore, STAT3/NF-κB decoy ODNs were shown to inhibit the expression levels of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β and IL-6, as well as the adhesion molecules VCAM-1 and ICAM-1 in atherosclerotic mice. In view of these results, STAT3/NF-κB decoy ODNs may be considered effective for the prevention and treatment of atherosclerosis, reducing inflammation through inhibition of the NF-κB signaling pathway.

The STAT family consists of seven members (STAT1, 2, 3, 4, 5A, 5B, 6) that transduce signals from various extracellular stimuli initiated by different cytokine families (35). STAT proteins exist in the cytoplasm in a latent form and are stimulated by tyrosine phosphorylation, which occurs when the JAK and Src families are activated (9). P-STATs form homo- or heterodimers that translocate to the nuclei and modulate the transcription of numerous target genes (36). Within the STAT family, STAT3 is an important transcription factor in both

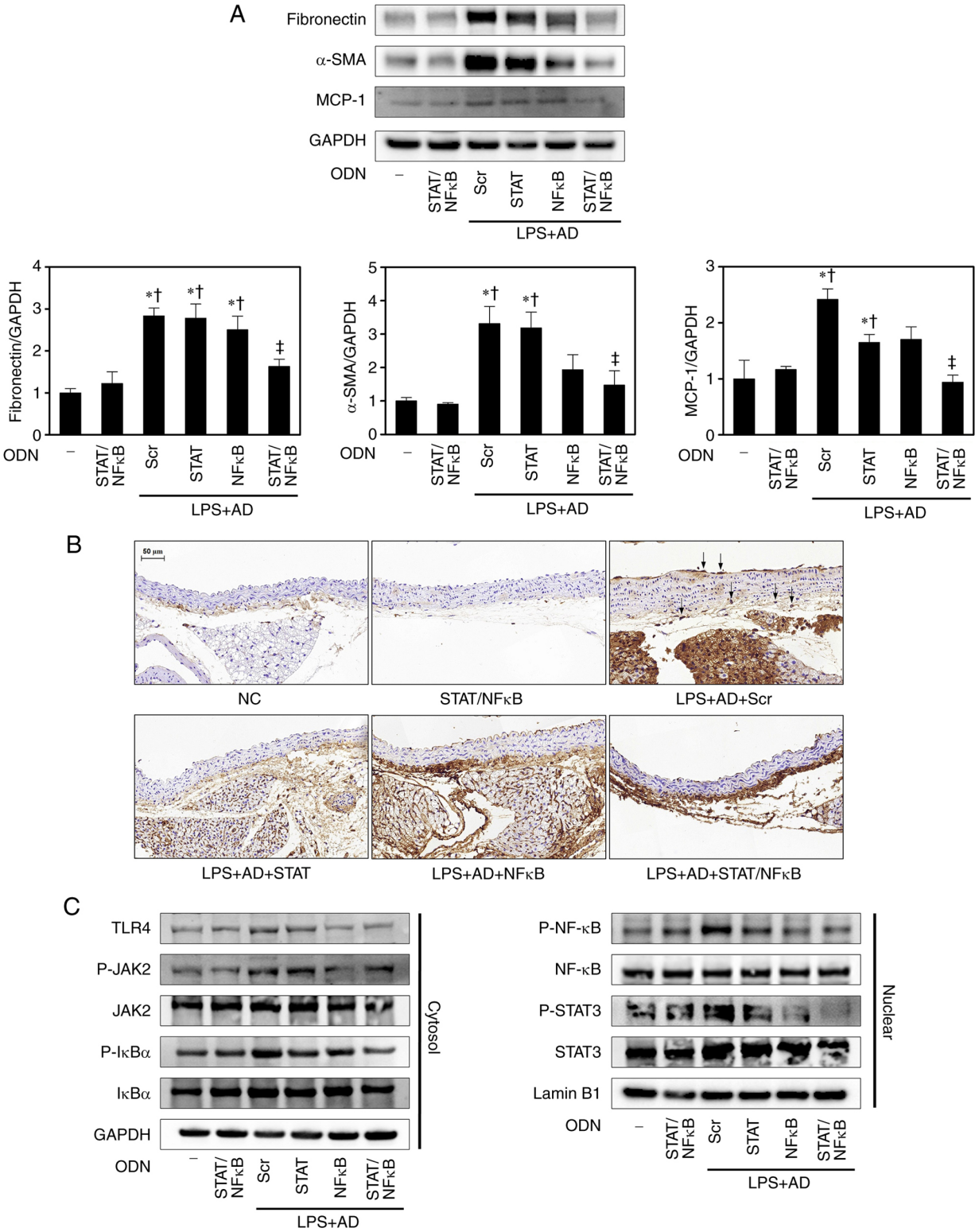

Figure 5. STAT/NF-κB decoy ODNs inhibit the expression levels of fibrosis-related proteins and the STAT/NF-κB pathway in atherosclerotic mice. (A) Western blotting was performed to detect the protein expression levels of fibronectin, α-SMA and MCP-1. The graph summarizes the semi-quantification of molecules of protein expression normalized to GAPDH (n=3). *P<0.05 vs. NC group; †P<0.05 vs. STAT/NF-κB group; ‡P<0.05 vs. LPS + AD + Scr group. (B) Immunohistochemistry images of the macrophage marker MOMA-2. Representative images from each group are shown (n=5). Arrows indicate areas of MOMA-2-positive cells. Scale bar, 50 μm. (C) Western blotting was performed to detect the protein expression levels of proteins in the STAT and NF-κB pathways (n=3). α-SMA, α-smooth muscle actin; AD, atherogenic diet; MCP-1, monocyte chemoattractant protein-1; NC, normal control; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; LPS, lipopolysaccharide; P-, phosphorylated; Scr, scramble; STAT, signal transducer and activator of transcription; TLR4, Toll-like receptor 4.

immunity and inflammation (37,38). It has previously been reported that STAT3 serves a crucial role in various diseases, including cancer, myocardial ischemic injury, cerebral stroke and obesity (1). In addition, it has been suggested that STAT3

may have a critical role in all pathological mechanisms of atherosclerosis, indicating that STAT3 could be a novel target of atherosclerosis therapies (1). The JAK/STAT intracellular pathway is essential in the regulation of leukocyte recruitment, foam cell formation, and the proliferation and migration of vascular SMCs (VSMCs), which are principal features of atherosclerosis (39-41). JAK2, STAT1 and STAT3 inhibition can reduce lesion size and neointimal hyperplasia (42). Based on these findings, it was observed in the present study that LPS-induced atherosclerotic mice fed an AD and treated with a STAT3/NF-κB decoy ODN exhibited reduced P-JAK2 and P-STAT3 protein expression levels. In conclusion, the present study may contribute to the application of STAT as a novel target for the treatment of atherosclerosis. The present study highlights the essential role of STAT3 in atherosclerosis and indicates that STAT3 inhibitors may be potential therapeutic agents for atherosclerosis.

The vascular inflammatory response includes complex interactions between inflammatory cells (lymphocytes, neutrophils, monocytes and macrophages), endothelial cells, VSMCs and the ECM (43). During chronic inflammation, ECM proteins and their fragments can regulate the migration of several types of cell, including endothelial cells, SMCs and monocyte/macrophages, which are well known to contribute to various stages of atherosclerosis (44). In particular, macrophages serve a decisive role at all stages of the progression of atherosclerotic lesions (45). Macrophages are the first inflammatory cells to invade atherosclerotic lesions, and secrete a wide range of cytokines and chemokines. NF-κB is a key transcription factor of macrophages that is required for inducing numerous inflammatory genes, including those encoding TNF-α, IL-1β, IL-6, IL-12p40 and cyclooxygenase-2 (46). Therefore, inhibiting inflammation and lipid deposition in macrophages may be an attractive therapeutic strategy for preventing atherosclerosis (47). In the present study, it was revealed that macrophage activation induced by an AD and LPS in atherosclerotic mice was markedly reduced by STAT/NF-κB decoy ODN.

In summary, the present study demonstrated that STAT/NF-κB decoy ODNs can suppress STAT and NF-κB signaling pathway activation in aortic tissues, and reduce VCAM-1, ICAM-1 and inflammatory cytokine expression. The present results also indicated that STAT/NF-κB decoy ODNs can mitigate atherosclerosis progression by magnifying ABCA1-mediated cholesterol efflux and relieving inflammation via inhibition of the TLR4 and STAT/NF-κB pathways. These results provide novel insights into the antiatherogenic molecular mechanism of STAT/NF-κB decoy ODNs and thus provide a novel method to prevent atherosclerosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJA, JBL and KKP contributed to the design of the study and wrote the paper. HJA, MGG, HG and SB carried out the experiments and analyzed the results. HJA and JL analyzed the data and edited the manuscript. HJA and KKP confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (Daegu, South Korea; approval no. DCIAFCR-181 204-27Y).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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