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STAT6 Upregulation Promotes M2 Macrophage Polarization to Suppress Atherosclerosis

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Background: Macrophages are highly heterogeneous and plastic cells that are involved in all stages of atherogenesis. They can undergo polarization by shifting between M1 and M2 functional phenotypes. However, the role of macrophage polarization and the molecular mechanism in modulating atherosclerotic plaque stability remain incompletely understood. Our study investigated the role of STAT6 in regulating macrophage phenotypes to affect atherosclerotic plaque stability.

Material/Methods: A murine atherosclerosis model with vulnerable plaques was induced with high-cholesterol diet and PCCP surgeries in ApoE$^{-/-}$ mice. Murine macrophages RAW264.7 treated with ox-LDL or IL-4 were used to simulate the *in vitro* process. pcDNA3.1(–)/STAT6-expressing vectors were transfected into RAW264.7 to evaluate its effect on cell polarization and the involved molecules.

Results: Unstable plaques presented significantly increased M1 markers (CD86 and iNOS) and less M2 markers (Arg-1 and TGF-β) than the stable plaques. Moreover, we found that STAT6 and p-STAT6 were greatly decreased in the vulnerable plaques and ox-LDL-induced macrophages, while their expression was elevated after IL-4 stimulation. The overexpression of STAT6 substantially reversed the ox-LDL-stimulated macrophage apoptosis and lipid accumulation. STAT6 upregulation promoted the differentiation of macrophage to M2 subtype as reflected by the increased expression of Arg-1 and TGF-β. Furthermore, we found that STAT6 overexpression activated the Wnt-β-catenin signaling by enhancing the translocation of β-catenin, while β-catenin suppression inhibited STAT6 overexpression-induced M2 polarization.

Conclusions: STAT6 facilitated atherosclerotic plaque stabilization by promoting the polarization of macrophages to M2 subtype and antagonizing ox-LDL-induced cell apoptosis and lipid deposition in a Wnt-β-catenin-dependent manner.

MeSH Keywords: Atherosclerosis • Macrophage Activation • Plaque, Atherosclerotic • STAT6 Transcription Factor • Wnt Signaling Pathway

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Background

Atherosclerosis (AS) is a chronic inflammatory disorder and a dynamic degenerative disease of the arterial wall, which affects many people around the world and is always associated with high mortality. It is characterized by the disturbance of cholesterol homeostasis in plasma and vascular cells, especially in resident macrophages in the subendothelial space in relation with excessive oxidation of circulating LDL [1,2]. The transition from asymptomatic to symptomatic disease mainly depends on sudden atherosclerotic plaque rupture and the subsequent thrombosis [3]. Once ruptured, atherosclerosis can give rise to acute atherothrombotic events such as myocardial infarction and stroke [3,4].

Macrophages play diverse roles in immune response due to their innate ability to adapt to the changing needs of the host [5]. In the pathogenesis of atherosclerotic plaques, circulating monocytes move into and reside in the subendothelium of vessel walls and transform into macrophages, which subsequently turn into foam cells after intaking oxidative LDL [6]. Macrophages are major source of foam cells and are decisive in the chronic inflammatory processes that drive atherogenesis. It is widely acknowledged that lesional macrophage apoptosis results in enforced plaque necrosis and instability, which increases the risk of thrombosis and even cardiovascular events. There are 2 phenotypes of macrophages in the plaque lesions – pro-inflammatory (M1) and anti-inflammatory (M2) subtypes – which are able to transform in response to the stimulation of serum lipids, lipoproteins, cytokines, chemokines, and small bioactive molecules. The classically activated M1 macrophage, stimulated by LPS and interferon-γ (IFN-γ), promotes the production of pro-inflammatory cytokines such as TNF-α, CCR7, iNOS, IL-1β, and IL-6. High concentrations of pro-inflammatory cytokines increase oxidative stress and apoptosis, and drive expression and activation of proteases during the initiation phase of atherosclerosis, in turn leading to plaque rupture and the resultant occlusive thrombosis [7]. The alternatively activated anti-inflammatory M2, induced by IL-4 and IL-13, is associated with tissue repair and endocytic clearance by secreting anti-inflammatory factors, including arginase-1 and Ym-1 [8,9].

The transcription factor family of Signal Transducer and Activator of Transcription (STAT) consist of 7 highly conserved members – STAT1, 2, 3, 4, 5A, 5B, and 6 – which play crucial roles in fundamental cellular processes, including cell growth, differentiation, development, apoptosis, immune response, and inflammation [10]. The enzyme STAT6 is a critical member of the STAT family, which is of importance in regulating cell differentiation and cytokine production. It was documented that STAT6$^{-/-}$ animals exhibited distinct IFN-γ and IL-4 cytokine expression profiles [11]. However, the role and underlying mechanism of STAT6 in modulating atherosclerotic plaque vulnerability remain incompletely understood. Our study intended to elaborate on how STAT6 regulates macrophage phenotypes to stabilize atherosclerotic plaques.

Material and Methods

Animal model

Twenty-four male ApoE$^{-/-}$ mice (18~22 g, 6–8 weeks old) on a C57BL/6Jbackground were obtained from Peking University (Beijing, China) and were used to establish the atherosclerosis model with unstable plaques according to a previously described method [12]. The animals were housed under moderate temperature and humidity with free access to food and clean water. All the mice were divided into 3 groups: control (ApoE$^{-/-}$ mice without any further treatment) (n=6), high-cholesterol-diet (HCD) fed ApoE$^{-/-}$ mice (n=9), and HCD-fed ApoE$^{-/-}$ mice underwent perivascular carotid collar placement (PCCP) surgery (n=9). Animals in the HCD group were provided food containing 1.25% cholesterol and 15% fat for 9 weeks. PCCP animals were provided with HCD food 2 weeks before surgery, followed by another 7 weeks of HCD for rapid induction of unstable plaques. The PCCP surgery was performed according to a previously described protocol [13]. In brief, the animals were anesthetized using intraperitoneal injection of pentobarbital sodium and fixed in an experimental platform. The common carotid arteries (CCA) of the mice were exposed and then were dissected free from the surrounding connective tissues. Collars with an inside diameter of 0.3 mm were deployed bilaterally around CCA, and their axial edges were approximated by the placement of 3 circumferential ties. Subsequently, the entry lesions were closed and the animals were returned to their cages for recovery. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University, and in compliance with the Guidelines for the Care and Use of Laboratory Animals.

Cell culture

Murine macrophage RAW264.7 cells were purchased from the American Type Culture Collection (ATCC), cultivated in DMEM medium (Hyclone, Logan, UT, USA) containing 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin A and streptomycin, and maintained in a 37°C humidified 5% CO_2 atmosphere. Cells were incubated with 50 mg/L ox-LDL for 24 h to induce the formation of foam cells. The M2 macrophage cell was induced by IL-4 (10 ng/mL) (Sigma, St. Louis, MO, USA) in murine macrophages for 24 h, in conjunction with transfection of siRNAs prior to the induction.

STAT6 overexpressing vectors and siRNA transfections

The cDNA sequence of STAT6 was synthesized based on its mRNA sequences by Sangon Biotech Co. Ltd. (Shanghai, China)

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and the synthesized STAT6 sequence was inserted into pcDNA3.1(–) vector to construct the recombinant pcDNA3.1(–)/STAT6-overexpressing vector. The negative control siRNA and siRNA specific for β-catenin or STAT6 were obtained from Invitrogen (Carlsbad, CA, USA). Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) was mixed with pcDNA3.1-STAT6 or STAT6/β-catenin siRNA at a 1: 1 ratio (v/v) at room temperature for 20 min. Then, the cells were incubated in serum-free DMEM with 60 nM pcDNA3.1-STAT6, STAT6/β-catenin siRNA, or their negative controls transfection mixtures for 6 h, then the medium was replaced with regular 10% FBS DMEM. After the transfection, the expression of STAT6 and β-catenin, as well as STAT6, were examined using Western blot analysis.

ELISA analysis and biochemical measurements

At the end of the HCD inducing procedure, a 5-ml blood sample was collected from all the mice at the marginal ear veins after they had been fasted for 12 h. The collected blood samples were centrifuged at 2000 r/min for 10 min and then the supernatants were stored at –80°C. The concentrations of plasma TNF-α, IL-6, and IL-8 were determined using commercially available ELISA kits (Jiancheng Biotech, Nanjing, China). Total serum cholesterol, triglycerides, and LDL were detected by diagnostic enzyme assay kits obtained from WAKO Pure Chemical Co. (Osaka, Japan). All the experimental procedures were performed in accordance with the manufacturers' protocols.

Histological and cell staining

After the HCD inducing procedure, all mice were euthanized by intravenous injection of pentobarbital, and their right CCA were excised and rinsed with sterile saline solution. Serial cryosections (5 μm) were prepared for histological analysis. Hematoxylin and eosin (H&E) staining was used to observe the morphology of atherosclerotic plaques. In brief, the sections were fixed in ice-cold acetone for 20 min and dried. After washing with phosphate-buffered saline for 1–2 min, the specimens were stained with hematoxylin followed by eosin. The lipid content in atherosclerotic plaques was visualized using Oil Red O (Sigma-Aldrich) staining for 10 min. The area of positive staining for Oil Red O was calculated as a percentage of total section area from 5–7 views for each mouse. For the Oil Red O staining of lipid droplets in ox-LDL-induced macrophages after STAT6-overexpressing vector or NC transfections, the cell cultures were centrifuged and the medium was discarded. Then, cells were washed with PBS and fixed with 4% formalin for 30 min. After staining with Oil Red O for 15 min, the morphology of foam cells was examined under an inverted microscope. For dual fluorescence staining, cryosections or cells were incubated with the mixture of primary antibodies from different species. Then, species-specific fluorophore-conjugated secondary antibodies were used to yield either a red (AlexaFluor 594) or green (AlexaFluor 488) fluorescent product at the site of the antigens. Images were acquired with a fluorescence microscope using ImageProPlus image analysis software (Media Cybernetics) and merged before further analysis.

RT-PCR

RT-PCR was performed to determine the expression of STAT6 and macrophages polarization markers in AS plaques and ox-LDL or IL-4 treated macrophages. Total RNA was extracted from frozen common carotid artery specimens and ox-LDL treated macrophages with TRIzol reagents (Invitrogen, Carlsbad, CA, USA). Then quality and quantity of the extracted RNA were analyzed by spectrophotometry. We reverse-transcribed 4.0 μg RNA to cDNA using an oligo-(dT) primer and reverse transcriptase (Qiagen, USA) according to the manufacturer's instructions. The amplification of the total cDNA was performed with the ABI Prism 7500 sequence detection system (PE Applied Biosystems, CA, USA) using a SYBR green real-time PCR Master Mix kit (Takara Biotechnology, Dalian, China) for 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. Specific RT-PCR primers were: STAT6 primers (F) 5'- TGA GGT GGG GAC CAG CCG G -3',(R)5'- GTG ACC AGG ACA CAC AGC GG -3'; iNOS: (F) 5'-CGA AAC GCT TCA CTT CCA A3', (R) 5' TGA GCC TAT ATT GCT GTG GCT 3'; CD86: (F) 5'-GGC CCT CCT CCT TGT GAT G-3', (R) 5'-CTG GGC CTG CTA GGC TGA T-3'; TGF-β: (F) 5'-AGA CGG AAT ACA GGG CTT TCG ATT CA-3', (R) 5-'CTT GGG CTT GCG ACC CAC GTA GTA-3'; arginase-1: (F) 5'-AAC ACG GCA GTG GCT TTA ACC-3', (R) 5'-GGT TTT CAT GTG GCG CAT TC-3'were used, with GAPDH primers (F: 5'-GCC AAA AGG GTC ATC ATC TC-3', R: 5'-GTA GAG GCA GGG ATG ATG TTC-3') as the internal control. Real-time PCR experiments for each gene were performed on 3 separate occasions.

Western blotting

Total proteins were extracted with RIPA (Beyotime, Jiangsu, China) agents from AS plaques and macrophages and the amount of proteins were determined using the bicinchoninic acid (BCA) method. Afterwards, the cell lysates were subjected to electrophoresis in sodium dodecyl sulfate (SDS)-PAGE and subsequently transferred to a PVDF membrane (Millipore, USA). Then, after the blockage with goat serum, the membrane was incubated with primary antibodies against STAT6, pSTAT6 and β-catenin (Abcam, Cambridge, UK) overnight at 4°C. After 3 washes with TBST, the membrane was probed with horseradish peroxidase-conjugated secondary antibody for an additional 2 h at room temperature. Blots were visualized using Amersham Western blot detection reagent (GE Healthcare, Piscataway, NJ, USA).

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Flow cytometry

For the examination of cell apoptosis, STAT6-vec or its matched negative control transfected macrophages were (1-5×10^5/well) were plated and incubated with ox-LDL for 24 h and were then digested with trypsin before being collected. Then, the apoptotic cells were determined by a Vybrant® Apoptosis Assay Kit (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) using Annexin V/PI double staining. The stained cells were analyzed by flow cytometry (BD, Franklin Lakes, USA).

For the analysis of CD86 and CD163-positive cells in response to STAT6 overexpression, 5×10^5 RAW264.7 were incubated with mouse monoclonal non-conjugated anti-CD86 and anti-CD163 antibodies (1: 1000) (Abcam, Cambridge, UK) for 30 min at 4°C in the dark. After washing with PBS 3 times, cells were labeled with secondary rat anti-mouse IgG-FITC (Abcam, Cambridge, UK) at 4°C for 30 min. Then, the labeled cells were washed, collected, and analyzed using a FACScan flow cytometry system.

Statistical analysis

Data are expressed as mean ± SEM. All statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL). Statistical comparisons were performed by one-way analysis of variance followed by the Student-Newman-Keuls test. $P<0.05$ was considered statistically significant.

Results

M1/M2 macrophages changes in AS plaques of ApoE$^{-/-}$ mice

In the current study, ApoE$^{-/-}$ mice underwent PCCP surgeries and were fed a high-cholesterol diet to induce the formation of vulnerable AS plaques in CCA. Compared to the control ApoE$^{-/-}$ mice, HCD-fed mice presented significantly elevated lesion areas and lipid deposition in the plaques, which had a profound increase in HCD-fed mice that underwent PCCP surgeries (Figure 1A). Moreover, we detected serum cytokines and lipid levels in these animals, and found that IL-6 substantially increased after HCD induction in ApoE$^{-/-}$ mice compared with the control, accompanied by the significantly increased total cholesterol (TC) and triglycerides (TG). TNF-α level remained unchanged and the IL-10 dramatically decreased after HCD induction. Moreover, we found that the HCD plus PCCP group had further increased TNF-α, IL-6, and ox-LDL, and reduced IL-10 level (Figure 1B). These data indicated that PCCP surgery facilitated rapid induction of formation of unstable plaques.

Furthermore, we examined the macrophage subtypes by determining the specific markers of M1 and M2 macrophages in the AS plaques of the animals using RT-PCR. The data revealed that vulnerable AS plaques had higher levels of CD86 and iNOS, and lower Arg1 and TGF-β than the stable plaques ($P<0.05$), indicating that macrophages resident in vulnerable AS plaques were prone to M1 polarization (Figure 1C).

STAT6 is decreased in AS plaques and ox-LDL-sensitized macrophages

To investigate roles of STAT6 in regulating AS plaque stability, we determined the expression of STAT6 in HCD-fed mice that underwent PCCP surgeries or not using RT-PCR and Western blot. RT-PCR results showed that STAT6 mRNA significantly decreased in mice with vulnerable AS plaques compared to that with stable plaques (Figure 2A). Moreover, we found that both STAT6 and phosphorylated STAT6 levels were substantially decreased in vulnerable lesions areas compared to the control (Figure 2B). Abundant M2 macrophages were identified in stable plaques using immunohistochemistry for CD163, accompanied by the expression of STAT6. Moreover, CD163 and STAT6 co-expressed macrophages at significantly higher frequencies in stable plaques compared to vulnerable plaques (Figure 3C). In addition, we determined the expression and phosphorylation of STAT6 in ox-LDL-stimulated murine macrophage RAW264.7 cells. The data showed that ox-LDL-induced foam cells presented less STAT6 and phosphorylated STAT6 than the cells without ox-LDL challenge (Figure 2D, 2E).

STAT6 upregulation promotes macrophages polarization towards M2 subtype

RAW264.7 cells were treated with IL-4 to induce the M2 polarization of the cells. We found that IL-4 exposure significantly promoted the expression and phosphorylation of STAT6 in macrophages compared to the control ($P<0.05$) (Figure 3A, 3B). To examine the role of STAT6 in modulating macrophage plasticity, we transfected the naïve RAW cells with STAT6 overexpressing vectors. After the transfection, the markers of the M1 and M2 subtypes were evaluated. The results demonstrated that the percentage of CD86-positive cells significantly decreased to 21.02±2.6% after STAT6 upregulation. However, CD163-positive cells increased to 82.6% ±10.9% after STAT6 upregulation (Figure 3C). In addition, IL-4-induced M2 differentiation was decreased after STAT6 silencing, accompanied by decreased CD163 and STAT6 positively co-localized cells (Figure 3D). These data revealed that STAT6 upregulation contributed to the induction of M2 polarization.

STAT6 upregulation antagonizes ox-LDL-induced foam cell formation and cell apoptosis

We further evaluated the effects of STAT6 upregulation on ox-LDL-induced foam cell expression and cell apoptosis, finding that upregulation of STAT6 largely suppressed the accumulation of lipid in ox-LDL-induced foam cells, as reflected by the

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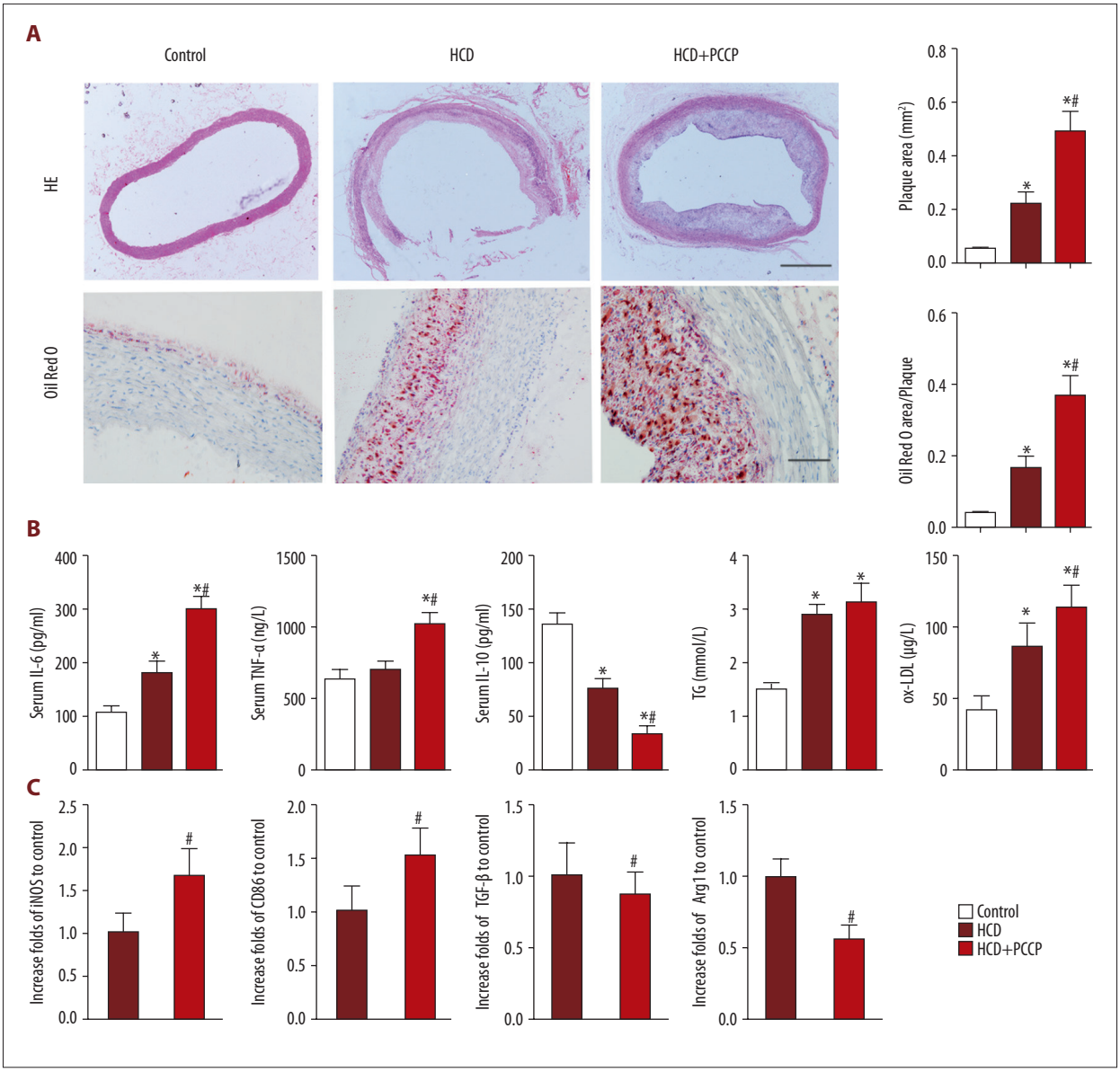

Figure 1. Macrophage polarization in atherosclerotic plaques of ApoE$^{-/-}$ mouse mice model. HCD-fed ApoE$^{-/-}$ mice underwent PCCP surgeries to rapidly induce the formation of vulnerable atherosclerotic plaques. (**A**) Right common carotid artery lesions were dissected and stained with HE (bar, 100 μm) or Oil Red O (bar, 50 μm) (n=6/group). Gross determination of plaque areas and Oil Red O-stained plaques in the 3 groups. (**B**) Serum levels of IL-6, TNF-α, total cholesterol, and ox-LDL in the animals. (C) RT-PCR determination of macrophage polarization markers in the plaques of each group. Data are presented as means ± SEM. * P<0.05, *vs.* control; # P<0.05, *vs.* HCD group.

reduced Oil Red staining (Figure 4A). In addition, cell apoptosis substantially decreased to 9.6±1.5% after the STAT6 overexpression in ox-LDL-stimulated macrophages (Figure 4B).

STAT6 actives Wnt-β-catenin to promote M2 macrophages polarization

It was reported that activation of Wnt signaling in macrophages by STAT6 promotes mucosal repair in murine inflammatory bowel disease (IBD) [14]. In the present study, we investigated whether Wnt-β-catenin cascades were also involved in STAT6-mediated M2 macrophages polarization, which then played roles in stabilizing the AS plaques. In STAT6-overexpressed macrophages, the expression of total β-catenin had no significant change, but nuclear β-catenin increased (Figure 5A). Moreover, treatment with IL-4 elevated the expression of total and nuclear β-catenin (Figure 5B). We further inhibited the expression of β-catenin by introducing its siRNA into the

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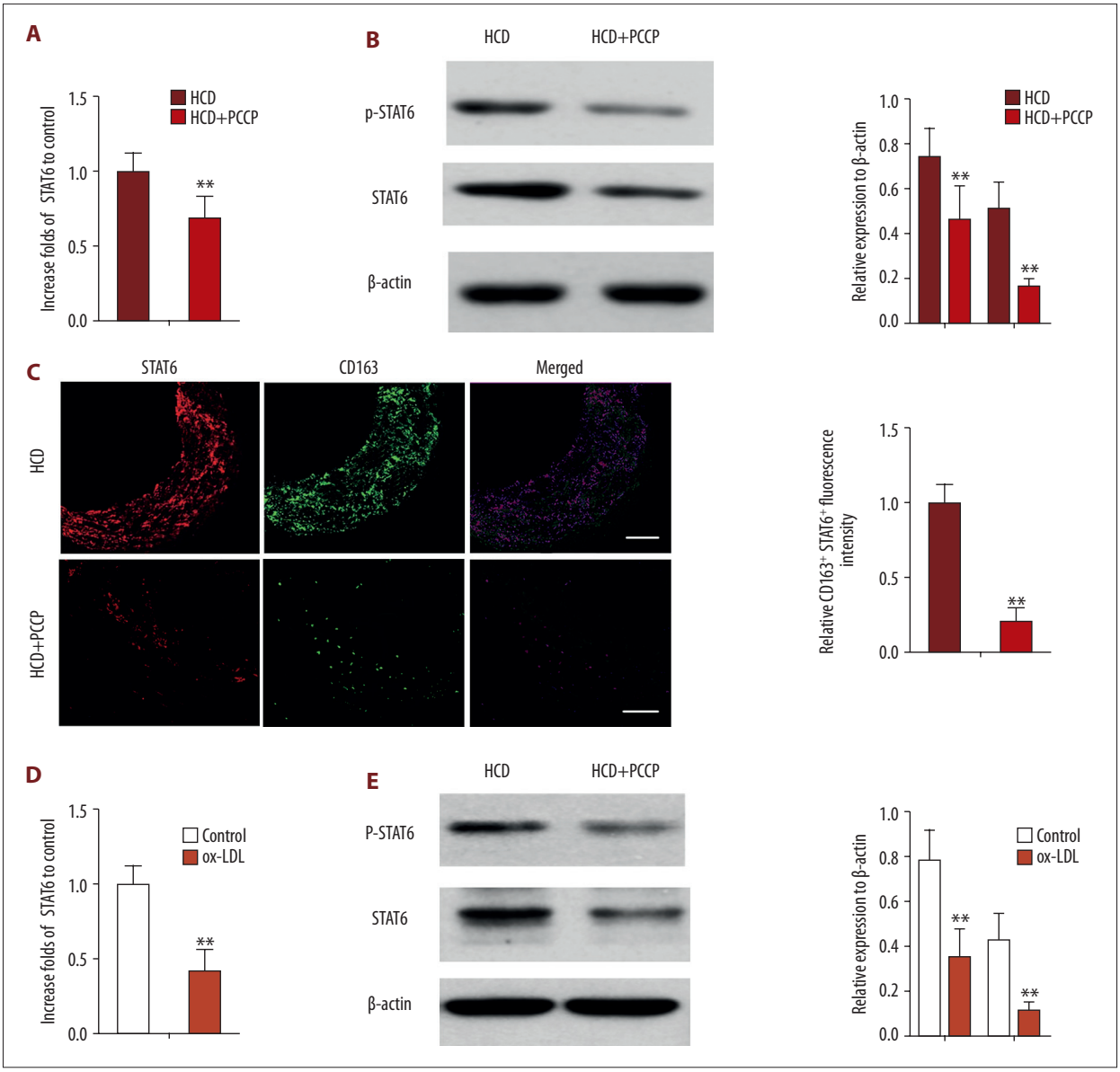

Figure 2. Expression of STAT6 in AS plaques and ox-LDL-sensitized macrophages. (**A**) RT-PCR analysis of STAT6 mRNA levels in plaques of HCD-fed ApoE$^{-/-}$ mice underwent PCCP surgeries or not. (**B**) Western blot determination of STAT6 and p-STAT6 in the plaques of the animals. ** P<0.01 *vs.* HCD group; (**C**) Immunofluorescence staining and quantification for CD163 and STAT6 as well as their co-localization in atherosclerotic plaques of ApoE$^{-/-}$ mice. Bar=50 μm, n=5. (**D**) Relative STAT6 expression before and after ox-LDL stimulation for 24 h. (**E**) Western blot analysis of STAT6 and p-STAT6 in the ox-LDL-treated RAW264.7 cells. ** P<0.01 *vs.* control group.

STAT6 overexpressed macrophages and evaluated the polarization of macrophages. The data showed that β-catenin inhibition greatly decreased the effects of STAT6 overexpression on the activation of M2 macrophages, as reflected by the decreased mRNA levels of Arg1 and TGF-β (Figure 5C). We also evaluated the role of β-catenin in IL-4-induced M2 transformation of macrophages, finding that β-catenin inhibition substantially suppressed the expression of Arg1 and TGF-β in IL-4-stimulated macrophages (Figure 5D).

Discussion

Atherosclerosis is a chronic degenerative disease that can result in cardiovascular disease, which is a leading cause of mortality and illness around the world. The clinical cardiovascular events are often caused by atherosclerotic plaques that may form flow-limiting stenosis or thrombus to reduce tissue perfusion. Our study demonstrated that STAT6 overexpression activated Wnt-β-catenin signaling to promote the polarization

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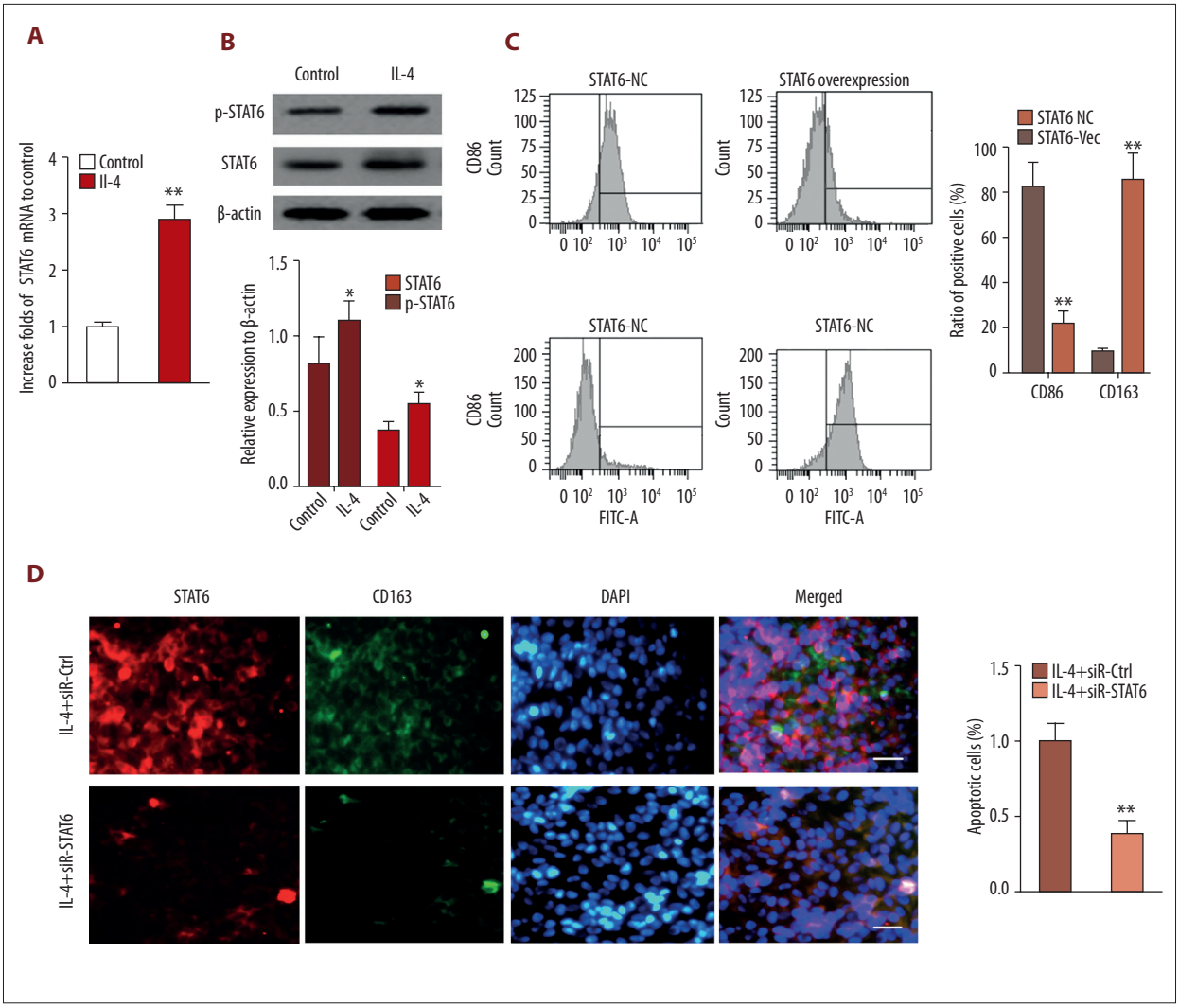

Figure 3. STAT6 upregulation promoted macrophages polarization towards M2 subtype. (**A, B**) Effects of IL-4 on the expression of STAT6 and phosphorylated STAT6 determined using RT-PCR or Western blot. * P<0.05, ** P<0.01 *vs.* Control. (**C**) pcDNA3.1/STAT6 plasmid and the negative control vector were transfected to RAW264.7 cells for 48 h, the cell surface markers were labeled with anti-CD86 and anti-163 markers, and then were analyzed using flow cytometry. ** P<0.01, *vs.* STAT6 NC. (**D**) Representative images of immune labeling for CD163 and STAT6 and their localization in STAT6 siRNA/NC-transfected macrophages followed by treatment with IL-4 for 24 h. Bar=250 μm, ** P<0.01, *vs.* IL-4+siR-Ctrl.

of macrophages to M2 subtype to stabilize the atherosclerotic plaques, which may be a promising approach to protect against acute coronary diseases (ACS) induced by plaque rupture.

Culprit plaque morphology and stability are closely associated with the occurrence of ACS and sudden cardiac death manifested as acute myocardial infarction and unstable angina. Clinical data showed that plaque erosion tends to present in women less than 50 years old, and the frequency of plaque rupture increased with each decade of life in older women [15]. Currently, atherosclerosis is recognized as an inflammatory disease with macrophages and T-lymphocytes playing dominant roles [16]. Macrophages are highly heterogeneous and plastic cells that play important roles in all stages of atherogenesis, ranging from the initiation of the necrotic core formation to plaque rupture. Growing evidence confirms that macrophages play critical roles in regulating stability of plaques. In advanced plaques, macrophage death contributes centrally to the occurrence of plaque necrosis, which underlies plaque instability that promotes plaque rupture and myocardial infarction[17,18]. Therefore, the modulation of macrophage function may act as a new target for repressing sudden rupture and the resultant unheralded cardiac events.

The circulating monocytes infiltrate and reside in the atherosclerotic plaques and transform to macrophages. When

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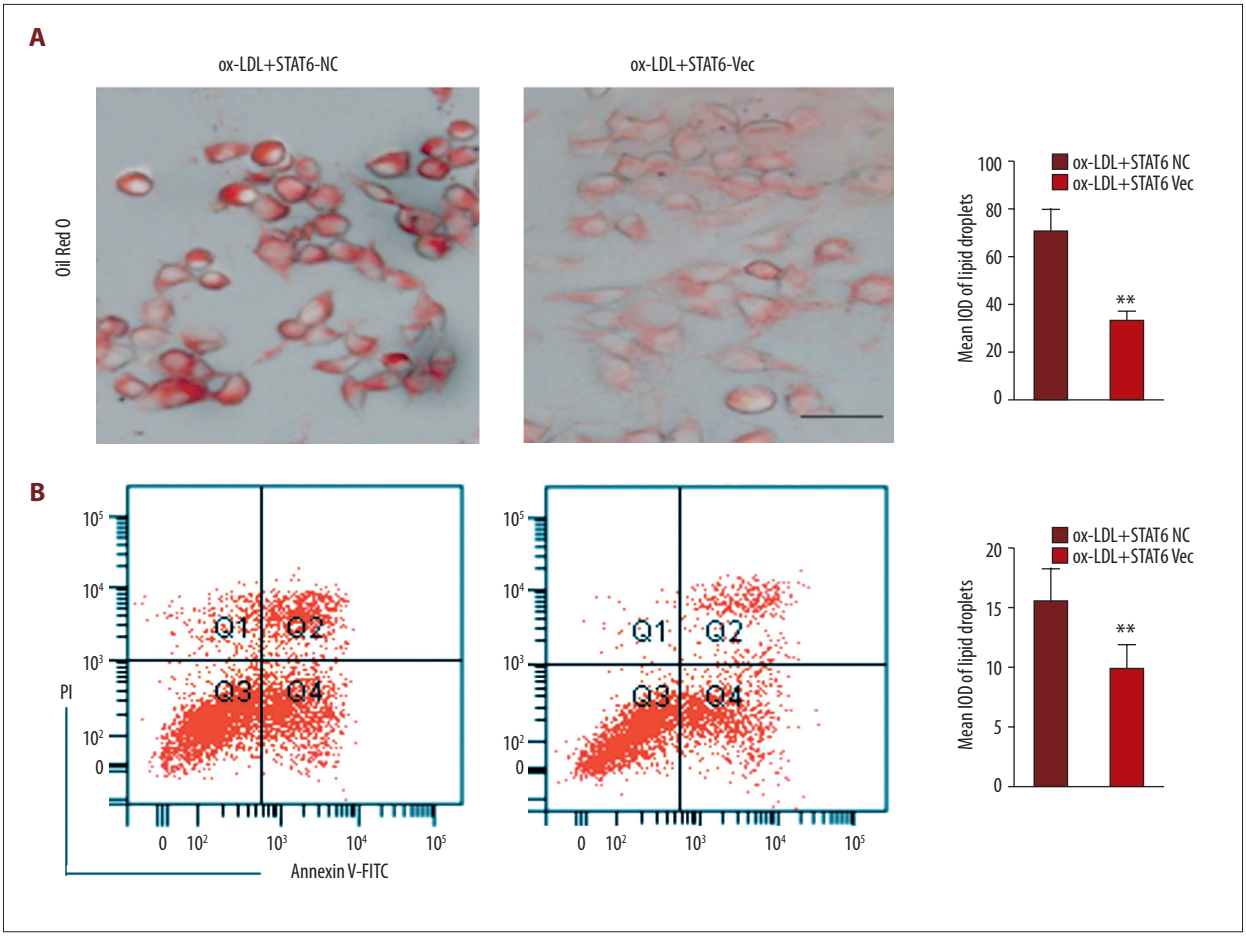

Figure 4. STAT6 upregulation suppressed ox-LDL-induced foam cell formation and cell apoptosis. (**A**) The pcDNA3.1/STAT6 or its negative control transfected macrophages were treated with 50 nM ox-LDL, and the lipid accumulation in the cells was determined using Oil Red O staining after 48 h of incubation. (**B**) Cell apoptosis in response to the ox-LDL was detected by flow cytometry. ** P<0.01, *vs.* ox-LDL+STAT6 NC.

sensing changes in the microenvironment, macrophages can shift between pro-inflammatory (M1) and anti-inflammatory (M2) functional phenotypes [19]. It was demonstrated that M2 phenotype macrophages induced by IL-4 dampen inflammation and promote tissue repair, in turn inhibiting the progression of atherosclerosis [16,20]. Moreover, the distribution of macrophage subsets within different plaque areas was markedly associated with cardiovascular vulnerability. A compelling clinical study documented that both M1 and M2 macrophages were present in human plaques; however, M2 macrophages were localized to more stable locations within the lesion, and the expression of M2 markers was inversly correlated with disease progression [21]. In addition, several studies reported that inhibiting M2 macrophage activation exacerbates cardiac dysfunction and plaque rupture following myocardial infarction in animal models [22,23]. Consistant with previous studies, we showed that M1 subtype macrophages were enriched in the unstable plaques in the murine model, while M2 macrophges were reduced.

STAT6 plays important roles in regulating cell differentiation and cytokines production. A recent study reported that STAT3 and STAT6 were potentially involved in regulating the M1 to M2 phenotypic switch [24]. It was also documented that STAT6$^{-/-}$ animals exhibited increased IFN-γ and reduced IL-4 cytokines expression in murine renal ischemia-reperfusion injury and murine neurocysticercosis [11,25]. IL-4 stimulation of macrophages promoted the phosphorylation of STAT6 via interleukin-4 receptor, providing initial instructions for alternative macrophage activation [26]. However, the role and underlying mechanisms of STAT6 in modulating atherosclerotic plaque vulnerability remain incompletely understood. In our study, we showed that STAT6 was decreased in the unstable plaques in mice, accompanied by the reduced M2 and elevated M1 macrophages markers. Moreover, the *in vitro* study revealed that STAT6 upregulation greatly promoted the M2 polarization and suppressed ox-LDL-induced cell apoptosis, indicating that STAT6 upregulation contributes to the stability of plaques by modulating macrophage differentiation. However, whether STAT6

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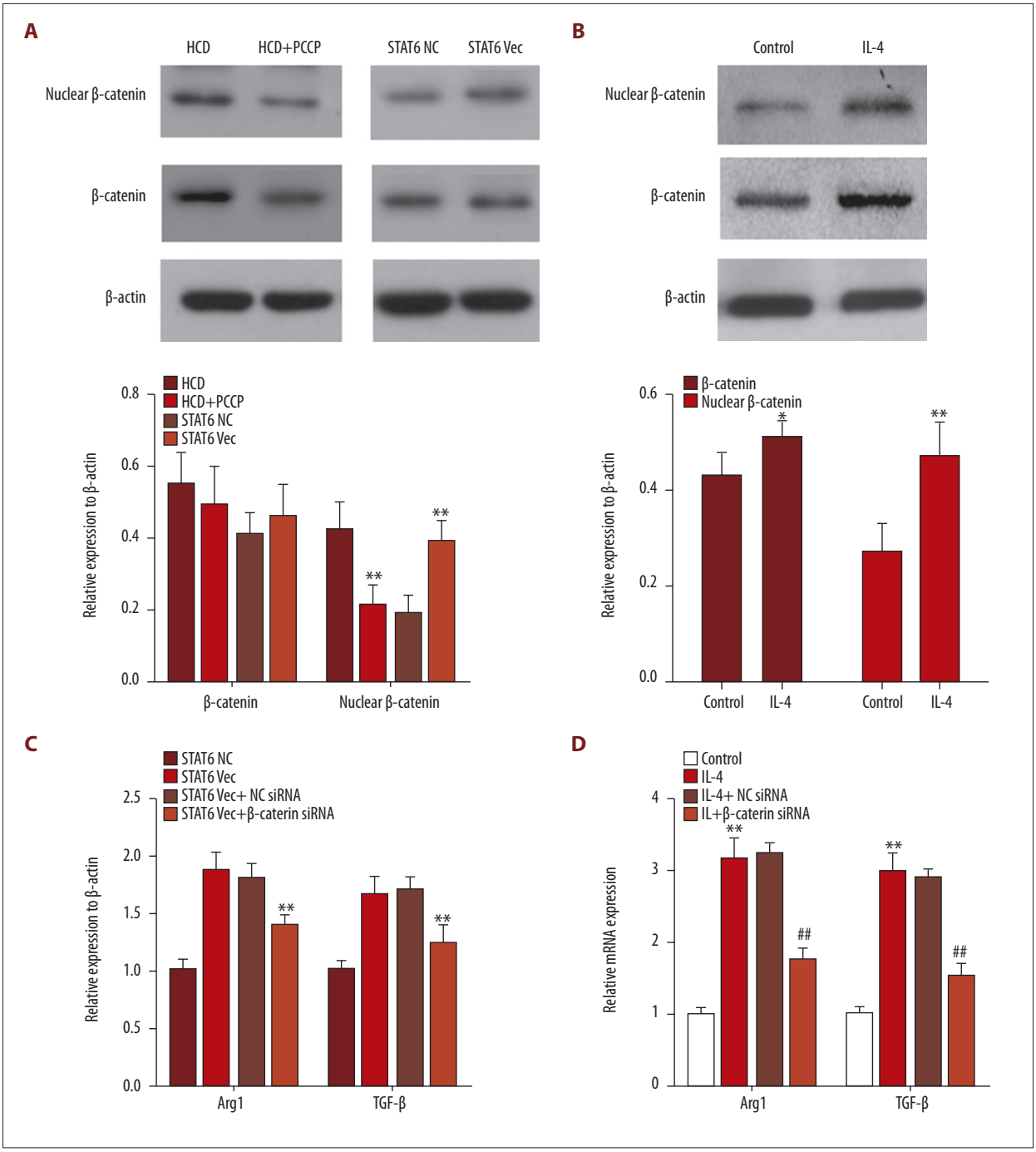

Figure 5. Wnt-β-catenin signaling was involved in STAT6-stimulated macrophages polarization. (**A**) Expression and quantification of total and nuclear β-catenin in stable and vulnerable plaques (n=5 for each group) and STAT6-overexpressing macrophages. ** P<0.01, compared with HCD or STAT6 vec. (**B**) Total and nuclear β-catenin levels in macrophages stimulated with IL-4 (10 ng/ml) for 24 h, * P<0.05, ** P<0.01 *vs.* control. (**C**) RT-PCR analysis of M2 macrophages markers in β-catenin silencing cells with STAT6 overexpression, ** P<0.01. (**D**) β-catenin siRNA or control transfected cells were exposed to IL-4 for 24 h, and the mRNA levels of Arg1 and TGF-β were examined using qRT-PCR, ** P<0.01 *vs.* control, ## P<0.01 *vs.* IL-4+NC siRNA.

is involved in the function of other components in plaques still needs further study. Currently, less is known, and no consensus has reached, regarding the underlying mechanism of STAT6 in regulating macrophage polarization. Kapoor confirmed that STAT6 implemented macrophage polarization via the dual catalytic powers of MCPIP [27]. Our study suggests

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that STAT6 activated Wnt-β-catenin to promote macrophage polarization to M2 subtype. It was reported that there was a cross-talk between JAK/STAT and Wnt/β-catenin signaling in leukemia cells, with β-Trcp playing key roles in this process [28]. Cosin-Roger et al. reported that IL-4 treated murine peritoneal macrophages overexpressed Wnt2b, Wnt7b, and Wnt10a in a STAT6-dependent manner, which contributed to mucosal repair in murine IBD [14]. Moreover, p-STAT3 repressed Gsk3β to increase β-catenin [29]. STAT-3 overexpression or activation significantly increased the level of β-catenin in pancreatic cancer cells [30]. However, more details concerning the mechanism of STAT6-Wnt/β-catenin signaling need to be described.

Conclusions

Our study demonstrated that M1 macrophages are abundant, but M2 cells were fewer in the vulnerable plaques in the AS animal model, accompanied by the reduced expression and phosphorylation of STAT6. The *in vitro* overexpression of STAT6 substantially promoted the polarization of macrophages to M2 subtype and antagonized ox-LDL-induced cell apoptosis and lipid deposition. STAT6 overexpression activated Wnt-β-catenin signaling by enhancing the nuclear translocation of β-catenin, and β-catenin suppression inhibited STAT6 overexpression-induced M2 polarization.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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