Original Article
Apolipoprotein B-100 peptide 210 antibody inhibits atherosclerosis by regulation of macrophages that phagocytize oxidized lipid

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Abstract: Immunization with peptides derived from apolipoprotein B-100 (ApoB-100) has been shown to ameliorate atherosclerosis in apolipoprotein E knockout (ApoE^{-/-}) mice. However, the exact mechanism underlying the therapeutic effects remains elusive. To shed light on this mechanism, we immunized ApoE^{-/-} mice that were fed a Western diet with either malondialdehyde-modified ApoB-100 peptide 210 (P210) emulsified in Freund's adjuvant or anti-malondialdehyde-modified P210 antibody (P210-Ab). Mice immunized with Freund's adjuvant or bovine serum albumin served as controls. Macrophages were incubated *in vitro* with oxidized low-density lipoprotein (ox-LDL) or ox-LDL plus P210-Ab. Our results show that P210-Ab promoted cholesterol efflux, inhibited lipid accumulation *in vitro*, and reduced plasma levels of high-sensitivity C-reactive protein (hsCRP), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). Furthermore, dramatically increased the expression of Fc receptors (FcR) on peripheral blood mononuclear macrophages, suggesting that the mechanism of phagocytosis of ox-LDL by mononuclear macrophages may rely more on FcR than the cluster of differentiation 36 (CD36) scavenger receptor with P210-Ab. Both *in vitro* and *in vivo*, P210-Ab triggered the promoter of ATP-binding cassette transporter A1 (ABCA1) to increase peroxisome proliferator-activated receptor alpha (α) activity and inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. In addition, P210-Ab significantly attenuated macrophage infiltration and markedly improved the stability of atheromatous plaque. In conclusion, the anti-atherosclerotic effect of P210-Ab is related to its preferential inhibition of inflammation and reversion of cholesterol transportation by altering the pathway by which macrophages phagocytize ox-LDL.

Keywords: P210, atherosclerosis, cholesterol efflux, inflammation, macrophages

Introduction

Atherosclerosis is a chronic progressive inflammatory disease of the arterial wall [1], for which endothelial dysfunction and oxidized low-density lipoprotein (ox-LDL) deposition in the arterial intima are among the most important initiating factors [2]. Despite LDL-lowering therapy, atherosclerosis is still a major cause of coronary artery disease, stroke, and other cardiovascular clinical events. The significant role of atherosclerosis in multiple diseases highlights the need for seeking additional anti-atherosclerosis therapies.

Macrophages play a key role in the pathogenesis of atherosclerosis [3]. They can recognize and engulf ox-LDL by scavenger receptor class A (SR-A), Toll-like receptors (TLR), or cluster of differentiation 36 (CD36) protein expressed on them, form foam cells, and then participate in initiating the inflammatory response. Moreover, the apoptosis and necrosis of macrophage foam cells are important events throughout the entire process of atherosclerotic plaque formation and development. One study verified that despite unaltered total serum cholesterol and triglyceride levels, TLR4 and TLR2 deficiency reduces intimal lipid accumulation by 75% and

45%, respectively, in apolipoprotein E knockout (ApoE$^{-/-}$) mice [4]. Another study confirmed that the absence of Fc receptors (FcR) increases atherosclerotic lesions five-fold in LDL receptor-deficient mice [5]. Moreover, macrophages phagocytizing ox-LDL less by SR-A, TLR, or CD36 can decrease inflammatory reaction activation. Hence, macrophages phagocytizing ox-LDL more by FcR may be a feasible approach to halt the development of atherosclerosis.

Macrophage foam cells can also transport intracellular cholesterol to extracellular locations or the liver by ATP-binding cassette transporter A1 and G1 (ABCA1/G1), scavenger receptor class B type I (SR-BI), and apolipoprotein A-I (ApoA-I) [6]. The transportation of extrahepatic cholesterol back to the liver for excretion, known as reverse cholesterol transport, may reduce the risk for atherosclerosis. Studies have shown that ABCA1 mutation results in a low level of high-density lipoprotein (HDL) and severe atherosclerosis [7], and macrophages lacking ABCG1 or SR-BI inactivation are able to promote the development of atherosclerotic lesions [8]. Analogously, atherosclerosis in ApoE$^{-/-}$ mice has been shown to be repressed by the up-regulation of ABCA1 and SR-BI [9]. Therefore, reverse cholesterol transport from macrophages is another strategy for the treatment of atherosclerosis. However, whether or not alternation of the pathway by which macrophages phagocytize ox-LDL is likely to facilitate its cholesterol efflux remains elusive.

Both the innate and adaptive immune systems play an important role in atherogenesis [10]. Similarly, immunoglobulin G (IgG) antibodies to ox-LDL are negatively correlated with cardiovascular disease in humans [11]. Other studies [12, 13] that may be relevant to protective immunity suggest that immunization using an apolipoprotein B-100 (ApoB-100)-related peptide inhibits atherosclerosis in ApoE$^{-/-}$ mice. These findings suggest that an antibody against ox-LDL or ApoB-100 peptide may be a new, effective, and preventive or therapeutic intervention for atherosclerosis. However, the specific mechanism of resistance to atherosclerosis with such an antibody remains unclear. This study was designed to investigate whether P210-Ab increases cholesterol efflux from macrophages and inhibits inflammation by altering the way in which macrophages phagocytize ox-LDL.

Materials and methods

Preparation of ApoB-100 peptide 210 (P210) and P210-antibody (P210-Ab)

The P210 and P210-Ab were prepared as described previously [14]. The peptides containing amino acids 3136-3155 (KTTKQSFD-LSVKAQYKKNKH) of ApoB-100, modified with malondialdehyde (MDA), were synthesized using a peptide synthesizer (PSSM-8, Shimadzu, Japan). The purity was determined to be 95% by high performance liquid chromatography. To prepare P210-Ab, Japanese white rabbits were immunized with P210 for 9 weeks. At week 9, blood was collected from these rabbits to determine P210-Ab titer by enzyme-linked immunosorbent assay (ELISA). The immunized rabbits with a high titer (>1:12000) were anesthetized and sacrificed by exsanguination from the common carotid arteries. Next, immunoglobulin (IgG) was prepared as described previously [14].

Animals

Seven-week-old male C57/BL6 ApoE$^{-/-}$ mice (Beijing HFK Bioscience Ltd., China) were used in this study. The mice were randomly divided into the active immunization group (AIG, n = 15) or the passive immunization group (PIG, n = 15). The PIG group was subdivided into a bovine serum albumin (BSA) group (n = 5) and a P210-Ab group (n = 10), and the AIG group was subdivided into the Freund-adjuvant (F-adjuvant) group (n = 5) and P210 group (n = 10). Animals were housed in a specific pathogen-free room in the experimental animal center of Tongji Medical College of Huazhong University of Science and Technology, at a constant temperature (22°C) with a 12-h light/dark cycle and given *ad libitum* access to food and water. The animals were given access to ordinary chewing diet to acclimate to the environment during the first week and then given a Western diet (0.15% cholesterol and 21% fat) until sacrifice at the age of 23 weeks. All protocols and procedures in this study were approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

Immunization protocol

Mice in the P210-Ab group were injected via the tail vein at weeks 1 (8 weeks of age), 4, 7,

10, and 13 with P210-Ab (200 µg/200 µl). Mice in the BSA group were intravenously treated with the same dose of BSA and served as the negative controls. Mice in the P210 group were immunized subcutaneously at different sites at 8 weeks of age with 200 µg/200 µl of P210 emulsified in Freund's complete adjuvant. A booster immunization with P210 emulsified in Freund's incomplete adjuvant was performed at weeks 4, 7, 10, and 13. Mice in the F-adjuvant group only received the same volume of Freund's adjuvant in the same manner and served as negative controls. Blood was obtained via the orbital venous plexus after overnight fasting and before immunization at weeks 1, 4, 7, 10, 13, and 16, and processed for the detection of antibody titer. Blood collected at week 1 was used as negative controls in analyses.

Cell culture

Human monocytic cell line cells (THP-1 cells) were obtained from Wuhan University and seeded onto fibronectin-coated 6-well tissue culture plates (Costar, Corning, NY, USA) with Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37.0°C. The THP-1 cells treated with 100 ng/mL phorbol-12-myristate-13-acetate (PMA; Enzo, NY, USA) for 72 h were differentiated into macrophages. Macrophages were divided into three groups: the control group, in which cells were incubated in RPMI-1640 medium for 24 h; the ox-LDL group, in which cells were incubated in medium supplemented with ox-LDL (50 mg/L) for 24 h; and the P210-Ab group, in which cells were incubated in medium supplemented with ox-LDL (50 mg/L) plus P210-Ab (100 µg/L or 200 µg/L) for 24 h.

Oil-red-O staining and fluorescent staining of macrophages

Macrophages in three groups were washed three times with phosphate-buffered saline (PBS), then washed once with 60% isopropanol diluted with distilled water for 10 seconds, and then stained with Oil-red-O as previously reported [15]. In fluorescent staining of macrophages, 5 µmol/L 22-(N(-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD)-cholesterol (Setareh Biotech, LLC, USA) was added to the ox-LDL and P210-Ab groups, and cells were washed three times with PBS, fixed with 4% paraformaldehyde for 20 min, and then stained with 4,6-diamidino-2-phenylindole (DAPI) in the dark for 30 s. Cells were observed under a light microscope (Olympus, Japan) and a fluorescence microscope (Olympus, Japan). Images were captured from at least five randomly selected fields for each group in at least three repeated experiments.

Cholesterol efflux assay

The cholesterol efflux assay was performed as described previously [16]. Briefly, after macrophages were treated with ox-LDL (50 mg/L) or P210-Ab (100 µg/L) plus ox-LDL (50 mg/L), NBD-cholesterol (5 µmol/L) was added to the ox-LDL group and P210-Ab group. Then, NBD-cholesterol-labeled cells were incubated in the fresh medium with ApoA-I. The fluorescence-labeled cholesterol was released from the cells into the medium, and fluorescence was measured with a microplate reader (Bio-Tek Instruments, Inc., VT, USA) at 469 nm (excitation wavelength) and 537 nm (emission wavelength) using a 96-well black plate. The following equation was used to determine the efflux rate from the fluorescence value (FI): cholesterol efflux rate = FI in induced efflux solution/(FI in induced efflux solution + FI in cell lysate solution) × 100%.

Plasma lipids analysis and body weight determination

After overnight fasting, 23-week-old mice were anesthetized by an intraperitoneal injection of sodium pentobarbitone (50 mg/kg). Blood samples were collected, and plasma was separated by centrifugation at 3000 rpm for 15 min at 4°C. The plasma levels of total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and triglyceride (TG) were measured using commercially available kits (Beijing-XinChuangYuan Biotech. Ltd., Beijing, China). The body weights of 23-week-old mice were also determined.

Measurement of antibody titer and cytokines

Antibody titers were measured by ELISA as described previously [17, 18]. Briefly, 96-well

ELISA plates were coated with antigen by adding 100 µl of coating solution plus 1-2 µg of P210 powder into each well. Next, each well was blocked with 200 µl of 2% BSA and incubated for 2 h at 37°C. One well was used as a blank control (100 µl PBST) and 2-4 wells were used as negative controls. Test serum was used in the remaining wells for control and experimental groups. Control groups were diluted as follows: (a) 2 µl serum + 198 µl diluent = 200 µl diluted serum (called S1 1:100), (b) 40 µl S1 + 160 µl diluent = 200 µl diluted serum (called S2 1:500), (c) 100 µl S2 + 100 µl diluent = 200 µl diluted serum (called S3 1:1000), (d) following the same pattern, dilutions of 1:2000, 1:4000, 1:8000, and so on were achieved. Experimental groups were treated as control groups and then incubated for 1.5 h at 37°C. After washing, 100 µl of secondary antibody solution was added, followed by 1 h of incubation at 37°C. After another washing, 100 µl of substrate solution was added and the solution was incubated for 3-5 minutes at room temperature (18-26°C) in the dark. The experimental reactions were terminated by adding 100 µl of stop solution (1 mol/L HCl). The plates were then read with a plate reader at a wavelength of 450 nm. Results were analyzed as follows: if the ratio of the optical density (OD) value of the test well to the average OD value of negative control groups was ≥2.1, the result was identified as positive and the corresponding minimum dilution factor was the antibody titer of the sample.

The levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) in the plasma were determined by ELISA (Neobioscience Technology CO. Ltd., China) according to manufacturer's instructions. The plasma level of high-sensitivity C-reactive protein (hsCRP) was measured according to the manufacturer's instructions (Elabscience Biotechnology CO. Ltd., China).

Tissue processing

The spleen was extracted, filtered through wire mesh, and washed in PBS for the separation of lymphocytes with a lymphocyte separation medium (LSM) (MP Biomedicals, CA, USA). The peripheral blood mononuclear cells (PBMCs) were suspended in PBS, followed by centrifugation. Cells were re-suspended in PBS for electrophoretic mobility shift assay (EMSA) and

flow cytometry. The liver was harvested, snap-frozen in liquid nitrogen, and stored at -80°C for real time-quantitative polymerase chain reaction (RT-qPCR) and western blotting. The heart was harvested as previously described [14]. Briefly, the heart and aorta were perfused with PBS from the apex of the heart. The base of the heart containing the aortic root was embedded in optimal cutting temperature compound (SAKURA Tissue-Tek, Japan) and sectioned in a slicing machine (Leica, Germany) at -20°C. Every fifth slide was collected for staining. The aorta was cleaned and opened along the axis.

Flow cytometry

The PBMCs were stained with antibodies against CD35-FITC (eBioscience, Fisher Scientific, PA, USA), CD64-PE (Becton Dickinson & Company, NJ, USA), and CD68-PE-Cy7 (eBioscience, Fisher Scientific, PA, USA) at room temperature for 30 min according to the manufacturer's instructions. Then, cells were washed with PBS twice and detected by flow cytometry using a FACSCalibur platform (BD Immunocytometry Systems, BD Biosciences, CA, USA).

Western blotting and real time-quantitative polymerase chain reaction

Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [17]. The primary antibodies used for western blotting included anti-ABCA1 (Novus Biologicals, CO, USA), anti-SR-BI (Novus Biologicals), anti-CD36 (Santa Cruz Biotechnology, TX, USA), anti-ABCG1 (EPITOMICS, CA, USA), anti-p-IκBα (Abcam, UK), anti-ACAT (Abcam), anti-p-p65 (Sigma-Aldrich, MO, USA), anti-GAPDH (Santa Cruz), and anti-β-actin (Sigma-Aldrich).

Total RNA was extracted from macrophages derived from THP-1 or liver tissues using Trizol (Takara Biotechnology, Japan) according to the manufacturer's instructions. Polymerase chain reaction amplification was then executed using QuantiTect SYBR Green PCR Kit (Takara Biotechnology, Japan) with specific primers (**Table 1**).

Electrophoretic mobility shift assay

An EMSA was performed as described previously [19]. Briefly, the nuclear proteins were

Table 1. Sequence of primers for real time quantitative PCR

Genes	Forward primer (5' to 3')	Reversed primer (3' to 5')
m-ABCA1	GCAATTATGTGGTCCCTGCT	ATGCCGATGAAGAGGTTCAC
m-ABCG1	GTACCATGACATCGCTGGTG	AGCCGTAGATGGACAGGATG
m-SR-BI	ATTCCCACGTATCGCTTCAC	GCTCCTTTGGGTTAGGGTTC
m-ACAT	GCATTCAGTGTGGTTGTGCT	TCCTCCTCCGTTGCAAATAC
m-β-actin	GGGAATGGGTCAGAAGGACT	CACACGCAGCTCATTGTAGA
m-PPARα	AGAAGTTGCAGGAGGGGATT	TCGGACTCGGTCTTCTTGAT
m-CD36	TGCAGGTCTATCTACGCTGTGT	GCGAAATATACACAGGCTTTCC
m-NF-κB	TAGAGGGAAGGATTCGAGCA	TGCTCTTAATGTGGCTGTCG
h-ABCA1	TCGGTGACTTATCCTGTGGTC	TTCTCAGATCTT GGCATTCGT
h-ACAT	AGTTGACAGCAGAGGCAGAG	GGATAAAGAGAATGAGGAGGG
h-GAPDH	GTCATCATCTCCGCCCCTTCTGC	GATGCCTGCTTCACCACCTTCTTG
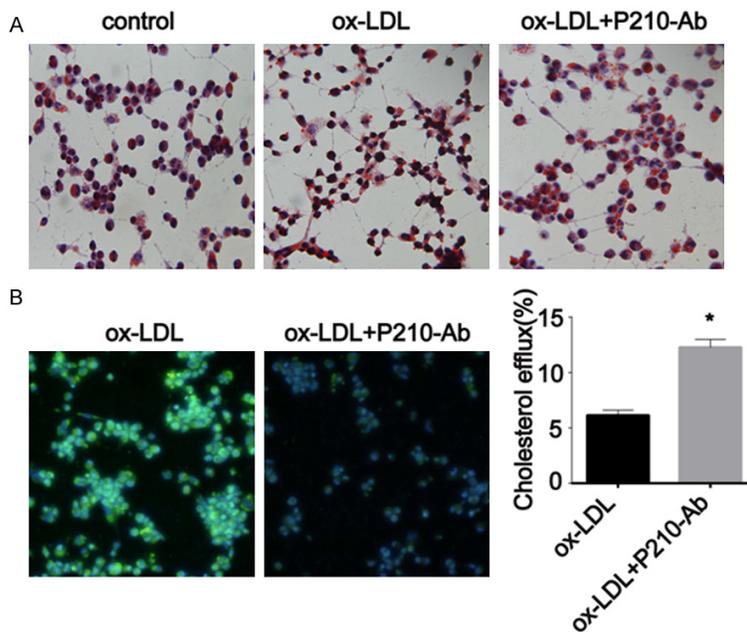
Figure 1. P210-Ab inhibited lipid accumulation and promoted cholesterol efflux *in vitro*. Data are expressed as means ± SEM from three independent experiments and normalized to the control group (or ox-LDL group). Macrophages were incubated with ox-LDL (50 mg/L) or ox-LDL (50 mg/L) + P210-Ab (100 μg/L) for 24 h. After incubation, cells were fixed and stained with Oil-red-O (A) or labeled with NBD-cholesterol (B). (A) Red refers to lipid and blue refers to nucleus. (B) The green fluorescence refers to cholesterol and blue fluorescence refers to the nucleus in the cholesterol efflux assay. The right column was quantitative analysis of cholesterol efflux rate. *P<0.05.

assay kit (Pierce, IL, USA). The activities of nuclear factor-κB (NF-κB) and peroxisome proliferator-activated receptor alpha (PPARα) in the nuclear extracts were measured with a Lightshift Chemiluminescent EMSA Kit (Pierce, IL, USA).

Oil-red-O staining

Frozen sections and aorta were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with Oil-red-O according to a standard protocol. The lipid deposition was observed as previously reported [15].

Immunohistochemistry

The cryosections (6 μm) from aortic sinus were stained with anti-CD68 antibody (Boster, China) for the identification of macrophages using a standard protocol [14]. Sections were then examined under a light microscope at a magnification of 100 ×.
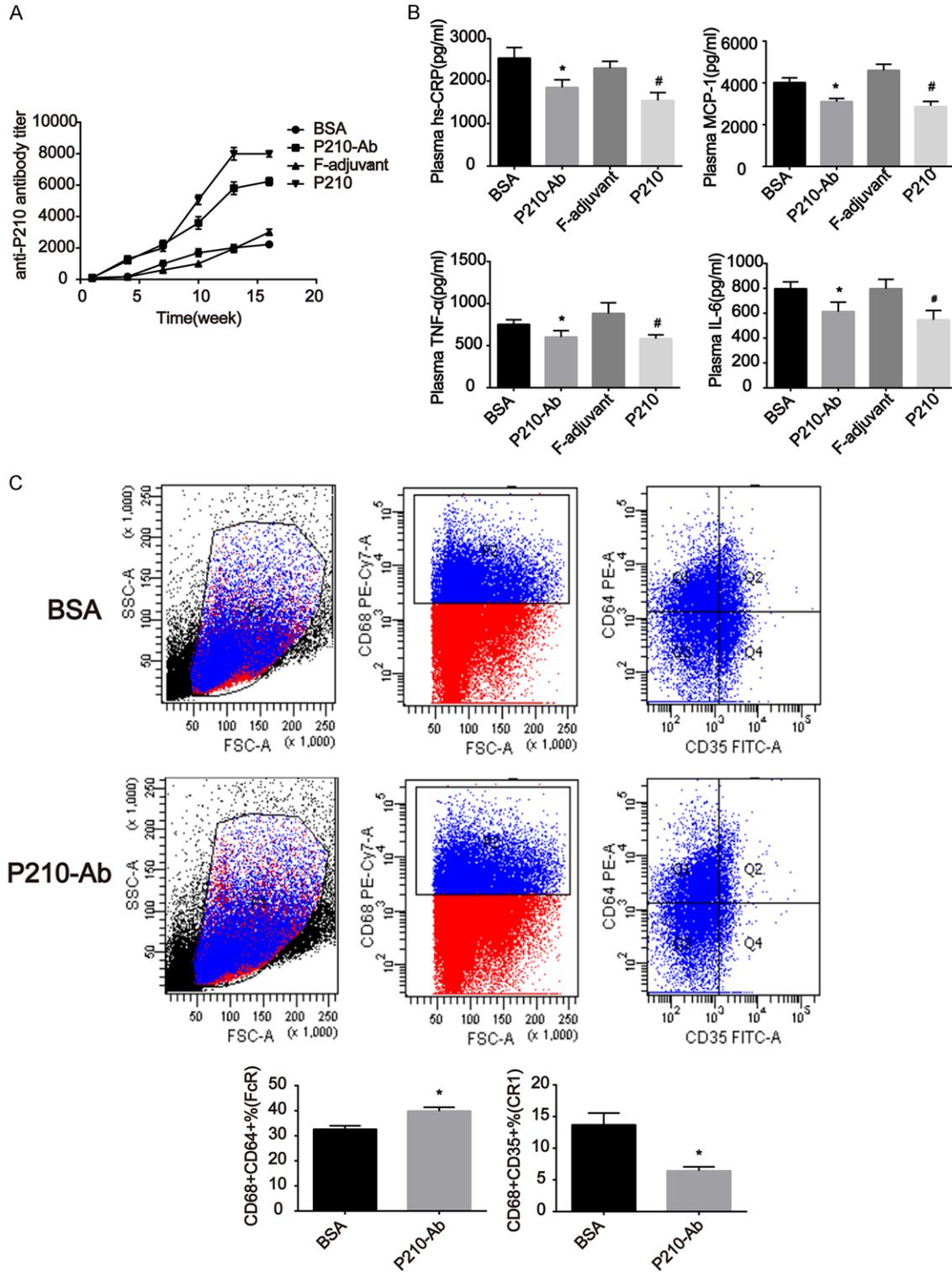
Masson staining

The collagen content of aortic sinus cryosections was assessed by standard Masson's trichrome staining [14]. Collagen was identified as blue tissues under a light microscope and examined at a magnification of 100 ×.

Statistical analysis

Data are expressed as mean ± standard error (SEM) and comparisons were done by one-way analysis of variance (ANOVA) among groups or Student's *t* test between two groups. The statistical analyses were performed using Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A value of P<0.05 was considered statistically significant.

extracted from PBMC in mice or macrophages stimulated with ox-LDL (12 h or 24 h) or ox-LDL plus P210-Ab (100 μg/L or 200 μg/L) according to the instructions of a commercially available kit (Nanjing KeyGEN Biotech. CO. Ltd., China). The concentration of nuclear proteins was determined with a bicinchoninic acid (BCA)

Figure 2. P210-Ab reduced plasma levels of hsCRP, MCP-1, TNF-α, and IL-6, and affected the expression of FcR (CD64) and CR1 (CD35) on PBMM. A. The titer of antibodies against P210 changes over time in ApoE^{-/-} mice. The titer of P210-Ab started to increase significantly after the first injection and continued to increase gradually with the following booster immunization in the P210 and P210-Ab groups but increased slowly in the BSA and F-adjuvant groups. B. P210-Ab affected the plasma levels of hsCRP, MCP-1, TNF-α, and IL-6 in ApoE^{-/-} mice at the age of 23 weeks. The plasma levels of hsCRP, MCP-1, TNF-α, and IL-6 were determined by ELISA. C. Immunization had an impact on PBMM of ApoE^{-/-} mice. The number of CD68^{+}CD64^{+} and CD68^{+}CD35^{+} cells in P210-Ab group and BSA group (control group), respectively, are shown. Data are expressed as mean ± SEM. *P<0.05 versus BSA group; #P<0.05 versus F-adjuvant group.

Table 2. Plasma levels of TC, TG, LDL-C, HDL-C, and body weight in ApoE$^{-/-}$ mice at the age of 23 weeks

Parameter*	PIG		AIG	
	BSA (n = 5)	P210-Ab (n = 8)	F-adjuvant (n = 5)	P210 (n = 9)
TC, mmol/L	26.01±2.740	24.11±1.690	24.55±2.223	19.92±2.095
TG, mmol/L	4.22±0.773	3.39±0.606	3.22±0.488	1.96±0.371
LDL-C, mmol/L	16.87±1.702	14.49±1.377	16.03±0.897	13.70±0.752
HDL-C, mmol/L	5.11±0.554	5.35±0.360	5.78±0.939	6.83±0.414
Body weight, g	30.10±0.966	29.33±0.553	30.68±0.893	29.91±0.526

Note: Data are expressed as mean ± SME. *TC: Total cholesterol; TG: Triglycerides; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol.

Results

P210-Ab promoted cholesterol efflux and inhibited lipid accumulation in vitro

The effects of P210-Ab on macrophages are shown in **Figure 1A**. Macrophages in the ox-LDL group had a larger variety of lipid droplets around the nucleus than the control group. However, macrophages in the P210-Ab group had fewer lipid droplets in the cytoplasm than the ox-LDL group. The lipid droplets of macrophages in the P210-Ab group were particles rather than large lipid crystals. The results in **Figure 1B** demonstrate that the lipids (green fluorescent) in macrophages in the P210-Ab group were significantly reduced in comparison to those in the ox-LDL group. The results in **Figure 1B** demonstrate that cholesterol efflux rate from macrophages incubated with ox-LDL plus P210-Ab was about two-fold elevated compared with that in ox-LDL group. These findings suggest that P210-Ab protects against the formation of macrophage foam cells by increasing the cholesterol efflux, which may involve antibody-mediated opsonization.

P210-Ab reduced plasma levels of hsCRP, MCP-1, TNF-α, and IL-6, and affected the expression of FcR (CD64) and CR1 (CD35) on peripheral blood mononuclear macrophages (PBMM)

As shown in **Figure 2A**, the titer of antibodies against P210 started to increase after the first injection and continued to increase gradually with the following booster immunization in the P210 and P210-Ab groups. After the third booster immunization, the titer reached a plateau (1:6000-1:8000). However, the titer in the

control groups (BSA group and F-adjuvant group) increased slowly, and the peak titer was 1:2000-1:3000 at week 16. In the absence of immunity induction, the antibody titer increased slowly and only a small increase was observed. The results in **Figure 2B** show that P210-Ab was able to reduce the plasma levels of hsCRP, MCP-1, TNF-α, and IL-6, thereby exerting an anti-inflammation effect. As shown in **Figure 2C**, the expression of FcR (CD64) on PBMM in the P210-Ab group was markedly increased compared with the BSA group, whereas the expression of complement receptor 1 (CR1/CD35) was significantly reduced. In addition, the body weight of all the ApoE$^{-/-}$ mice was similar, and the activity or behavior remained unchanged in all the groups. P210-Ab treatment did not change the plasma levels of TC, TG, and LDL-C in the AIG and PIG groups (**Table 2**). These results indicate that P210-Ab may be able to promote ox-LDL phagocytosis via FcR but not CR1 in macrophages, thereby reducing the initiation of inflammation.

Effects of P210-Ab on NF-κB (p-p65/p-IκBα), ABCA1, ACAT, and PPARα in cells and ApoE$^{-/-}$ mice

Western blotting after P210-Ab treatment showed that the relative protein expression of ABCA1 and ACAT markedly increased but p-p65 and p-IκBα decreased significantly (**Figure 3A**). Analysis by RT-qPCR showed that the relative mRNA expression of ABCA1 and ACAT increased significantly after P210-Ab treatment (**Figure 3B**). Also, the EMSA results shown in **Figure 3C** indicate that the nuclear PPARα activity increased, but NF-κB activity decreased after P210-Ab treatment. Similar results were also observed *in vivo*. The relative protein expression of ABCA1, ABCG1, and SR-BI in the P210-Ab group increased markedly, but p-p65 and CD36 expression declined compared to the BSA group. This result was also observed between the P210 group and the F-adjuvant group (**Figure 3D**). In P210-Ab or P210 group, the relative mRNA expression of SR-BI, ABCG1,
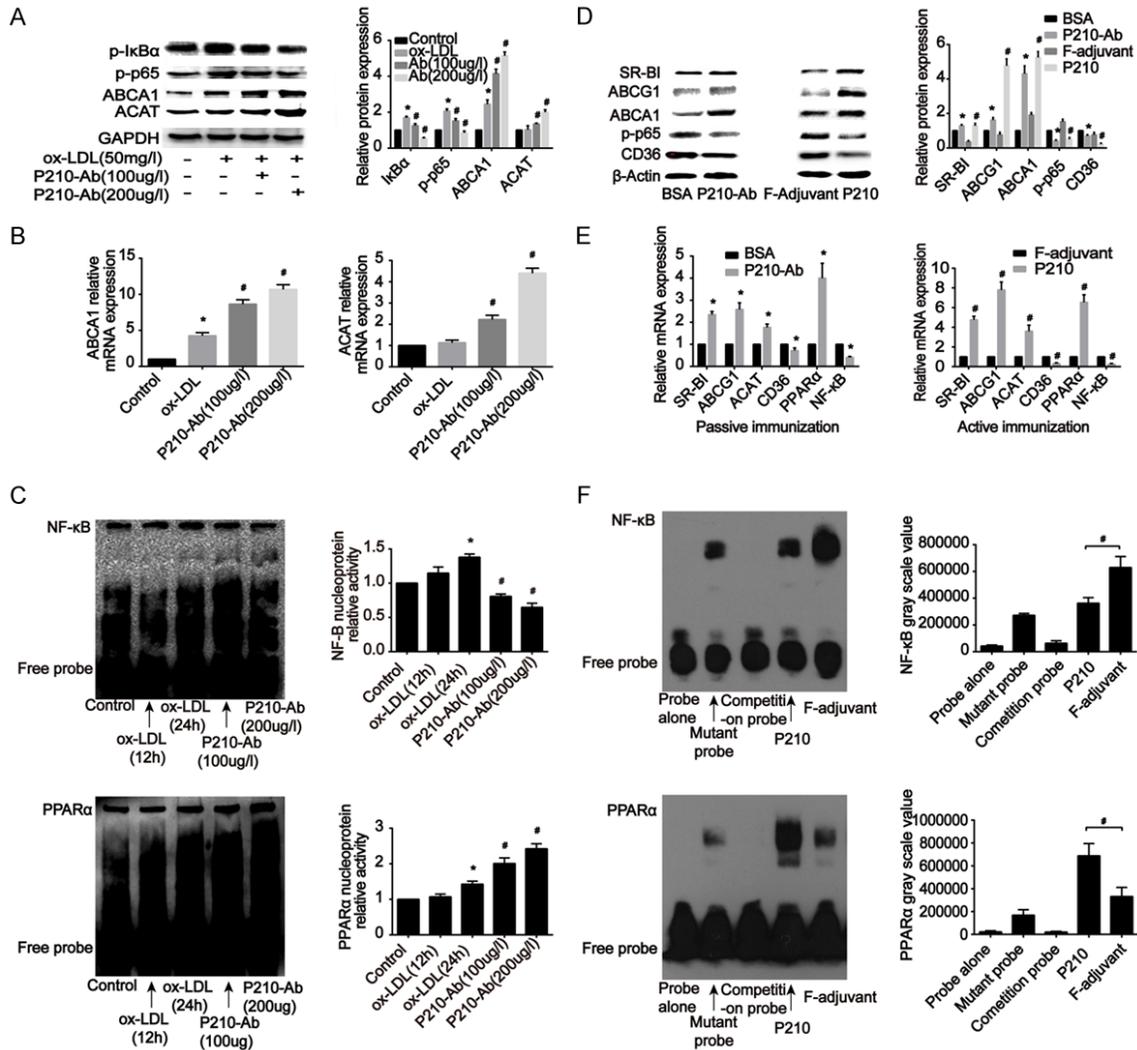
Figure 3. Effects of P210-Ab on NF-κB (p-p65/p-IκBα), ABCA1, ACAT, and PPARα in cells and ApoE$^{-/-}$ mice. (A-C) Macrophages derived from THP-1 were incubated with or without ox-LDL (50 mg/L), ox-LDL (50 mg/L) + P210-Ab (100 μg/L), and ox-LDL (50 mg/L) + P210-Ab (200 μg/L). (A) Protein expression of ABCA1, p-p65, p-IκBα, and ACAT detected by western blotting. (B) The relative mRNA expression of ABCA1 and ACAT detected by RT-qPCR. Representative immunoblots from three separate experiments were shown, the expression of proteins and mRNA were normalized to that of GAPDH. (C) Activities of NF-κB and PPARα in macrophages (EMSA). (D) Relative protein expression of SR-BI, ABCG1, ABCA1, p-p65, and CD36 detected by western blotting in liver tissues of different groups. (E) The relative mRNA expression of SR-BI, ABCG1, ACAT, CD36, PPARα, and NF-κB detected by RT-qPCR in liver tissues of different groups. (F) Activities of NF-κB and PPARα in PBMCs of ApoE$^{-/-}$ mice in different groups (EMSA). Data are expressed as mean ± SEM. *P<0.05 versus control; #P<0.05 versus ox-LDL group (A-C); *P<0.05 versus BSA, #P<0.05 versus F-adjuvant group (D-F).

ACAT, and PPARα increased significantly but NF-κB and CD36 declined markedly compared to the BSA or F-adjuvant group, respectively (**Figure 3E**). Results of EMSA show that nuclear PPARα activity increased but NF-κB decreased significantly (**Figure 3F**), which was also observed *in vitro*. These results indicate that P210-Ab upregulates ABCA1, ABCG, and SR-BI expression via a PPARα pathway and inhibits the activation of NF-κB and the expression of CD36 and p-p65.

P210-Ab reduced atherosclerotic lesion and affected the content of macrophages and collagen in the aortic sinus

The ApoE$^{-/-}$ mice were fed with a western diet for 15 weeks with or without P210-Ab treatment. The en face lesions along the aorta were significantly reduced in ApoE$^{-/-}$ mice treated with P210-Ab or P210 compared to BSA or F-adjuvant control groups, respectively. In the PIG group, the P210-Ab subgroup showed a
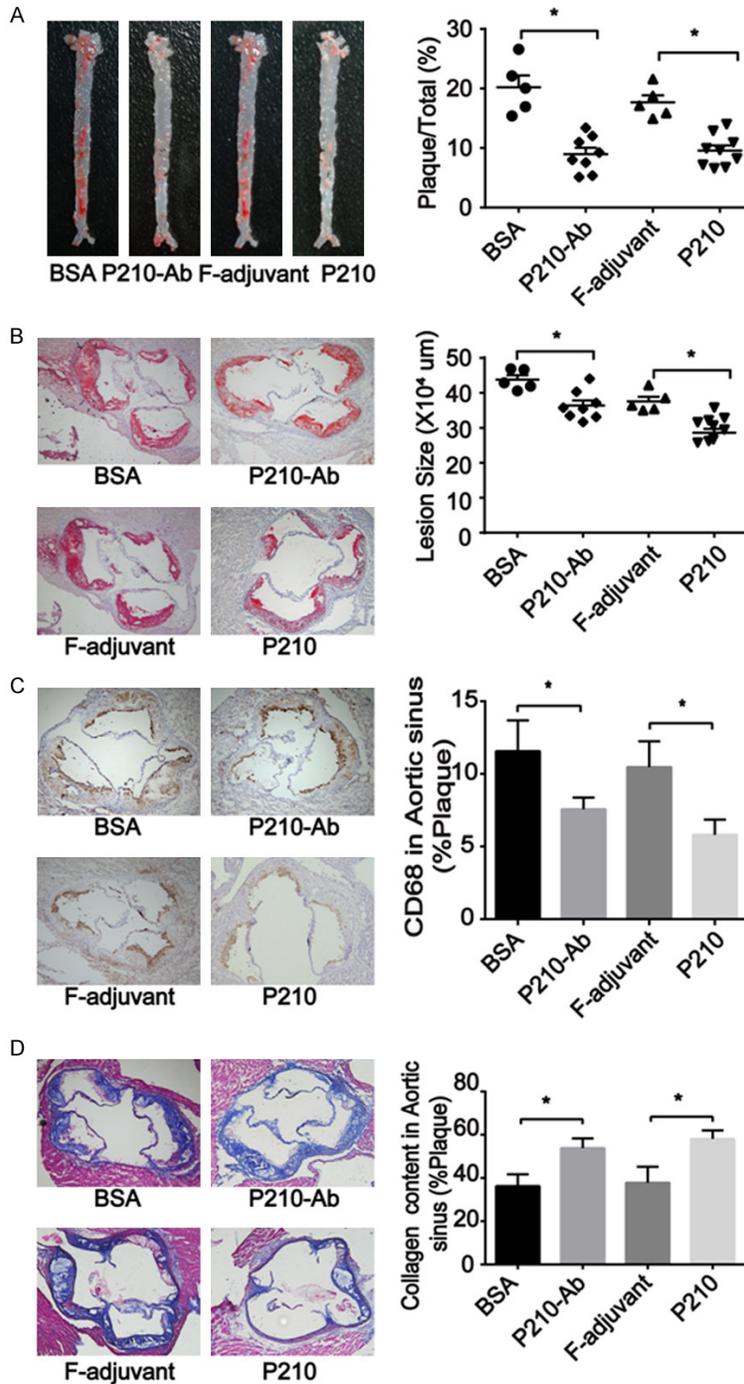
Figure 4. P210-Ab reduced atherosclerotic lesion and affected macrophages and the content of collagen in the aortic sinus. (A, B) The inhibitory effect of P210-Ab on the atherosclerosis of ApoE$^{-/-}$ mice at the age of 23 weeks. Representative images of en face (A) and aortic sinus sections (B) by Oil-red-O staining. Quantitative analyses of en face and aortic sinus sections (100 ×) located in the right column. (C, D) Representative images of aortic sinus section after immunohistochemistry for CD68 (C) (100 ×) and Masson staining (D) (100 ×). Quantitative analyses of CD68 and collagen in the aortic sinus. Data are expressed as mean ± SEM; n = 5, 8, or 9; *P<0.05.

55.7% reduction in the extent of aortic atherosclerosis compared to the BSA group, whereas the P210 subgroup was reduced by about 46% compared to the F-adjuvant group (**Figure 4A**). Quantitative analysis of atherosclerosis lesion was performed by measuring the Oil-red-O positive area in the aortic sinus. The plaque areas in the aortic sinus of ApoE$^{-/-}$ mice treated with P210-Ab (36.38±1.45 × 10^4 μm^2) or P210 (28.61±1.1 × 10^4 μm^2) were significantly reduced compared to the plaque areas in control mice treated with BSA (43.81± 1.3 × 10^4 μm^2) or F-adjuvant (37.55±1.32 × 10^4 μm^2) alone (**Figure 4B**). To elucidate the effect of P210-Ab on the content of macrophages and collagen in the aortic sinus, immunohistochemistry and Masson staining were performed. The plaques in the aortic sinus immunized with P210-Ab or P210 had, respectively, 19.7% or 53.3% fewer macrophages than the BSA or F-adjuvant control group (**Figure 4C**). Interestingly, the P210-Ab and P210 groups had more collagen than their respective BSA and F-adjuvant control groups. The collagen content of the aortic sinus in the P210-Ab group increased by 21.1% compared to the BSA group and that in P210 group increased by 25.6% compared to the F-adjuvant group (**Figure 4D**).

Discussion

Chronic inflammation and immune responses are present in the entire process of atherosclerosis [20, 21]. Our study indicates that anti-ApoB-

100 peptide 210 antibody (P210-Ab) might inhibit the inflammatory response of atherosclerosis by the NF-κB pathway. In addition, our results show that P210-Ab enhanced the PPARα-ABCA1/ABCG1/SR-BI pathway responsible for cholesterol metabolism, leading to the outward transportation of cholesterol from macrophages. A novel finding of our study is that P210-Ab inhibits the expression of CD36 (SR-B) on mononuclear macrophages, but increases the expression of the Fc receptor on mononuclear macrophages. This result suggests that mononuclear macrophages may phagocytize ox-LDL more by FcR than by CD-36 due to the P210-Ab-mediated regulation. Overall, we can reasonably conclude that P210-Ab is likely to delay the development of atherosclerosis by inhibiting inflammation and promoting reverse cholesterol transportation by altering the way in which macrophages phagocytize ox-LDL.

Ox-LDL is mainly phagocytized through CD36 and SRA scavenger receptors in macrophages. *In vitro* studies have shown that about 75-90% of ox-LDL is phagocytized by macrophages in CD36- and SRA-dependent manners [22]. However, the scavenger receptor of macrophages lacks a negative feedback mechanism, which leads to continuous phagocytosis of ox-LDL in macrophages until they die [22]. Our study showed that, after immunization, the IgG isotype of P210-Ab was produced at a higher titer in ApoE$^{-/-}$ mice. The Fc receptor on macrophages also increased, but CD36 expression decreased. In the presence of P210-Ab, ox-LDL that was phagocytized by the Fc receptor increased in macrophages, but ox-LDL phagocytized by CD36 decreased. Therefore, altering the way in which cholesterol is processed in macrophages as well as macrophage foaming, might change the necrosis fate of macrophages in the process.

Increasing the reverse cholesterol transportation to the liver for metabolism is effective in inhibiting the development of or even reversing atherosclerosis [23]. Our results showed P210-Ab was able to promote cholesterol efflux, but did not reduce blood levels of LDL-C and TC in ApoE$^{-/-}$ mice treated with P210-Ab. ABCA1/G1 and SR-BI are important receptors in reverse cholesterol transportation. Studies have shown that the loss of ABCA1, ABCG1, or SR-BI will result in the accumulation of cholesterol in

macrophages, thereby promoting the atherosclerosis [7, 8, 24]. P210-Ab could up-regulate the expression of ABCA1/G1 and SR-BI and increase PPARα activity. Increasing ABCA1/G1 expression may facilitate the cholesterol efflux in macrophages, and increasing SR-BI expression may promote the HDL-mediated cholesterol efflux from the arterial wall as well as the hepatic uptake of HDL-C. This effect is similar to that of EP80317 and quercetin, which can enhance the activity of PPARγ-LXR signaling pathway in macrophages, promote ABCA1 expression, and increase cholesterol efflux, thereby exerting inhibitory effect on atherosclerosis. In our study, the mRNA and protein expression of ACAT in macrophages significantly increased after immunization. This increase may in turn increase cholesteryl ester and reduce the formation of cholesterol crystals which can cause toxic effects and accelerate the development of atherosclerosis.

Inflammation plays an important role in the pathogenesis of atherosclerosis, and the NF-κB pathway is a crucial factor involved in inflammation [25]. P210-Ab can inhibit the mRNA and protein expression of NF-κB and the expression of p-p65. The serum levels of pro-inflammatory cytokines hsCRP, TNF-α, IL-6, and MCP-1, which are affected by NF-κB [25], were reduced after immunization in our study. These results suggest that P210-Ab can significantly inhibit the inflammatory response primarily by NF-κB pathway and thereby possibly delay the development of atherosclerosis.

Our findings of decreased cholesterol deposition, reduced macrophage activity and increased collagen content in the aortic sinus plaque observed in ApoE$^{-/-}$ mice treated with P210-Ab are consistent with the findings of a study by Fredrikson et al. [26]. The ratio of atherosclerotic plaque to total aortic area was also reduced after treatment with P210-Ab. However, the body weight and blood lipid levels (LDL and TC) remained unchanged after immunization in this study. Thus, the anti-atherosclerotic effect of P210-Ab may not completely rely on the lowering of plasma cholesterol lipid, which is different from the mechanism underlying the anti-atherosclerotic effect of statins and PCSK9 [27].

In summary, our study for the first time demonstrates that P210-Ab can effectively inhibit ath-

erosclerosis through inhibiting inflammation and up-regulating PPARα-ABCA1/G1 and SR-BI expression to reverse cholesterol transportation. These actions may be related to the P210-Ab-mediated regulation in addition to the neutralization reaction. P210-Ab may alter the metabolic pathway of ox-LDL in macrophages by altering the way in which the macrophages phagocytize ox-LDL. In the presence of a specific antibody against P210, the mechanism of ox-LDL metabolism in macrophages may be altered from "pattern recognition receptors mediated phagocytosis-inflammation initiation-atherosclerosis formation" to "specific antibody intervention-promoting cholesterol efflux-curbing atherosclerosis". This alteration of the mechanism of lipid processing can inhibit inflammation, promote cholesterol efflux, avoid excessive lipid load, and improve macrophage survival. All these factors contribute to the suppression of atherosclerosis. The specific mechanisms for this proposed sequence of lipid processing remains unclear and requires further research.

Acknowledgements

Disclosure of conflict of interest

None.

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