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Oxidized low-density lipoprotein induces M2-type differentiation of macrophages to promote the protracted progression of atherosclerotic inflammation in high-fat diet-fed ApoE -/- mice

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ABSTRACT

In this study, the significance of oxidized low-density lipoprotein (ox-LDL) in promoting the progression of atherosclerosis was investigated by inducing the differentiation of macrophages into the M2 subtype within a high-fat diet-induced ApoE -/- mouse model. The study also evaluated the effects of β2-AR agonists and blockers on this process. Ox-LDL was found to have significantly promoted the differentiation of macrophages into the M2 type and induced related functional alterations. Furthermore, it activated the pyroptosis pathway and encouraged the release of lactate dehydrogenase. The administration of β2-AR agonists intensified these processes, while β2-AR blockers had the opposite effect. In animal experiments, the model group displayed elevated numbers of M2-type macrophages beneath the aortic root intima, an increased rate of plaque destruction, and the formation of atherosclerotic plaques compared to the control group. The SAL (Salbutamol) group exhibited even more severe plaque development than the model group. Conversely, the ICI (ICI118551) group demonstrated M2-type macrophage levels comparable to the control group, with a higher plaque destruction rate than controls but significantly lower than the model group, and no atherosclerotic plaques. These findings suggest that ox-LDL promoted the differentiation of recruited monocytes into M2-type macrophages, leading to a shift in the inflammatory response from M1 to M2 macrophages. This alteration resulted in the persistence of atherosclerotic inflammation, as M2-type macrophages were prone to cell membrane rupture (such as pyroptosis), contributing to the continuous recruitment of circulating monocytes and heightened inflammatory reactions within atherosclerotic plaques. Consequently, this process fueled the progression of atherosclerosis.

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Introduction

Atherosclerosis (AS) is a chronic inflammatory disease of the vascular wall caused by the innate immune response driven by myeloid monocytes and macrophages (1, 2). The mechanism of its persistent course has not been clarified yet. Normally, macrophages are mainly in the form of mononuclear cells derived from bone marrow or resident macrophages from tissues derived from the yolk sac and embryonic liver, which exist in the blood and all tissues, respectively, and maintain the steady state of the body. The function of macrophage and its corresponding phenotype are highly plastic and heterogeneous. Macrophages can generally be simply divided into two large subpopulations with distinct functions, namely, classical activated pro-inflammatory type (M1) and alternating activated anti-inflammatory type (M2). M1 type and M2 type can be transformed with each other. Therefore, M1 type and M2 type of macrophages are also considered as two extremes of a series of functional states of macrophages. The specific phenotype and function of macrophages in tissues are only in a certain M1-type or M2-type polarization state between M1 type and M2 type, which is not necessarily in the extreme state of polarization, and the degree of polarization state can be adjusted. The specific phenotype of activation is not only related to the tissue source but also related to the microenvironment of macrophages (1). It is generally believed that the phenotype of tissue-resident macrophages defaults to M2 type (3), and circulating monocytes are the precursor cells of M1 type macrophages (4).

Type M1 and type M2 macrophages were distributed in AS plaques. The correlation between macrophage polarization and atherosclerotic plaque vulnerability has been studied. In the early stage of AS lesions, it is generally believed that the main entrance to AS plaques is circulating monocytes differentiated into M1-type macrophages (4), and monocytes are the key source of early foam cells (5). However, studies have found that the phenotype of macrophages accumulated in the fatty streak plaques of early AS lesions is M2 type (4). Based on the anti-inflammatory and repair characteristics of M2-type macrophages, especially in animal models with AS regression, there is a phenomenon that M1-type macrophages convert to M2-

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type macrophages, the number of M2-type macrophages is increased, and the ratio of M2/M1 is increased. The traditional mainstream view holds that increasing the number of M2-type macrophages in AS plaques is an important method to induce the progression of AS plaques (4, 6-10). However, the early lipid-striation lesion rich in M2-type macrophages is not a marker of AS plaque prognosis. On the contrary, such lesions will progress to the typical AS plaque, and the reason is still unknown. The clinical use of drugs that promote the conversion of macrophages to M2 type has also failed to achieve the desired effect (11).

Pathological studies have found that normal repair of damaged tissues depends on the fact that newly recruited monocytes in the damaged area firstly polarize to the M1 type, that is, M1 type macrophages must clear away the pathogenic factors through inflammatory reaction in the early course of disease. After the pathogenic factors are completely cleared, the recruitment of monocytes by the lesion stops, and M1 type macrophages in the lesion differentiate to M2 type, which together with M2 type macrophages from other sources complete the repair (3, 12-14). Repair is delayed if the effects of the causative agent cannot be eliminated, or if the procedural order in which type M1 and type M2 macrophages must be strictly followed is disturbed during the inflammatory response. At present, it has been found that biological pathogenic factors such as tuberculosis, tumors, and hepatitis C virus can promote the M2 type differentiation of macrophages and disturb the procedural order that M1 and M2 type macrophages must strictly follow in the early stage of inflammation-repair/regeneration process, i.e., the inflammatory reaction stage, before the biological pathogenesis disappears, resulting in the persistent progress of the inflammatory course (15-17). Ox-LDL has lipid toxicity on macrophages, and at the same time, it can induce macrophages to differentiate into M2 type (18-20). Therefore, at the early stage of the inflammation-repair/regeneration process of AS lesions, it is necessary to conduct research on whether ox-LDL can also cause the body to fail to repair AS lesions by disrupting the procedural order that M1-type and M2-type macrophages must strictly follow, resulting in the persistent progression of the disease course.

Materials and Methods

Reagents and antibodies

Fetal bovine serum (Gibco®, 10099141C, Rockville, MD, USA) were purchased from Thermo fisher Scientific (Waltham, MA, USA); RPMI-1640 (PM150122) sugar-free basal medium and penicillin-streptomycin antibody (PB180120) were purchased from Procell (Wuhan, China); Phobos ester (PMA, P6741) was purchased from Solarbio Life Sciences (Beijing, China); Salbutamol (HY-B1037) , Metoprolol (HY-17503), Propranolol (HY-B0573B) and ICI118551 (HY-13951)were purchased from MedChemExpress (Monmouth Junction, NJ, USA); Ox-LDL (YB-002-1) was purchased fromYiyuan Biotechnology (Guangzhou, China); Reagent kits for TNFα (ML077385) and IL-10 (MM-0066H1) were purchased from MLBIO (Shanghai, China); TRIzon Reagent (CW0580S) and Ultrapure RNA Ultra Pure RNA extraction kit (CW0581M) were purchased from CWBIO (Beijing, China); HISCRIPT II Q RT Super Mix for QPCR (+GDNA Wiper) (R223-01) from VAZYME (Nanjing, China); 2×SYBR Green PCR Master Mix (A4004M) was purchased from Lifeint (Xiamen, China) and Oil Red O staining kit from LeageneBiotechnology (Beijing, China).

Cell culture and macrophage preparation

The human acute monocyte cell line (THP-1 cell) was provided by the Procell (Wuhan, China) and cultured in the 1640 medium (Procell, Wuhan, China) containing 10% fetal bovine serum (Procell, Wuhan, China) at 37°C and 5% CO_2. The medium was changed once every two days. The cell growth was microscopically observed, and the color of culture medium, cell morphology, growth, and pollution condition should be carefully noticed. The cells were then cryopreserved at -80°C and recovered before use.

THP-1 cells were adjusted to a density of 10^6/ml under stable growth conditions. PMA (Shanghai Baiyi Biology Co., Ltd., Shanghai, China, final concentration of 160 nmol/L) was added to the medium and THP-1 cells were cultured at 37°C and 5% CO_2 for 24 hours. After induction and differentiation, THP-1 cells adhere to the vial wall and form macrophages.

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Cell grouping and medication

Cell grouping

The macrophages were randomly divided into a Blank Control group, an experimental control group and different drug treatment groups.

Macrophage medication after LPS+IFN-γ induction

Adding the same amount of culture medium as the control group into the blank control group; The drug treatment group was pretreated with different concentrations (low dose, ld, 1 μmol/L; Medium dose, md, 10 μmol/L; High-dose, hd, 50 μmol/L) salbutamol or ICI118551 (different concentrations of metoprolol and propranolol were added in addition to ICI118551 based on known clinical efficacy for further evidence. Metoprolol doses were the same as ICI118551, and the low dose of propranolol was 0.1 μmol/L, the medium dose was 1 μmol/L, and the high dose was 10 μmol/L) were pretreated with the corresponding drugs for 1 hour. All the groups except the blank control group were added with a final concentration of 10 pg/mL LPS and 20 ng/mL IFN-γ and the cultures were continued for 6 hours before the supernatant and cells were collected.

Macrophage medication after IL4 induction

The mice were grouped as before. After pretreatment with the corresponding drugs for 1 hour, IL-4 with a final concentration of 20 ng/mL was added into all groups except for the blank control group. After continuous culture for 24 hours, the supernatant and cells were collected.

Detection of TNFα or IL-10 by Enzyme-linked immunosorbent assay

The supernatant collected by different groups and the standard substance was added into the corresponding

wells of 100 L/ well, cultured at 37°C for 90 min, rinsed three times, cultured at 37°C in biotin-anti-human TNF-α or IL-10 working solution (100 L/well) for 60 min, rinsed three times, cultured at 37°C in biotin-peroxidase working solution (ABC, 100 L/ well) for 30 min in the dark, rinsed five times, cultured in TMB developing solution (90 L/ well) for 30 min and then measured the absorbance at 450 nm in different experiments.

Reverse transcription fluorescent quantitative PCR assay

The cell supernatant was taken and dissolved with 1 ml TRIzol reagent for 10 min, followed by extraction with chloroform, 400 L isopropanol, and centrifugation. The supernatant was discarded and sample RNA was collected for reverse transcription into cDNA. RNA concentration was detected by fluorescent quantitative PCR. Reaction conditions: Pre-denaturation at 95°C for 30 s, followed by denaturation at 95°C for 5 s and annealing at 60°C for 30 s for 40 cycles, with β-action as the internal reference for each amplification. The relative expression levels of iNOS and Arg-1 in the samples were analyzed by the $2^{-\Delta\Delta CT}$ method. The fluorescent quantitative PCR primer are as follows:

Arg-1: F: 5'-ACCATAGGGATTATTGGAGC-3';
R: 5'-TGTCATTAGGGATGTCAGCA-3';
iNOS: F: 5'-CGTGTTTCACCAGGAGATG-3';
R: 5'-CAGCATACAGGCAAAGAGC-3';
β-actin F: 5'-AGCGAGCATCCCCCAAAGTT -3';
R: 5'-GGGCACGAAGGCTCATCATT -3'

Detection of CD80 and CD206 by flow cytometry

The cells were digested and collected in EP tubes, washed with PBS buffer three times, and blown into individual cells. After the cell count was adjusted to 2×10^6/ml, a proper amount of primary antibody was added, and the cells were mixed evenly and incubated for 30 min; in the dark condition. Then, 1 ml PBS was added into each tube, mixed evenly, centrifuged at 1000 rpm for 5 min, used to remove the supernatant, and PBS was used to reset the suspension and the procedure was repeated three times. A proper amount of fluorescently labeled secondary antibody was added and incubated for 30 min; in the dark condition. After the supernatant was removed, 1 ml PBS was added into each tube, mixed evenly, and centrifuged at 1000 rpm for 5 min for three times, to remove the supernatant; then 500 μL PBS reset suspension was added into each tube and tested on the computer.

Ox-LDL promotes M2-type differentiation of macrophages

Cell grouping and drug therapy

After THP-1 cells were induced into macrophages, they were randomly divided into a blank control group, an experimental control group, or a drug treatment group. The cells in the medication group were pretreated with a medium dose (10 μmol/L) of SAL or ICI118551 for 1 hour, and all groups except the control group were incubated with a final concentration of ox-LDL (China Guangzhou Yiyuan Biotechnology Co., Ltd.) at 50 mg/L for 24 h. The supernatant and cells were collected.

Enzyme-linked immunosorbent assay

TNFα and IL-10 were determined by ELISA as described above.

Reverse transcription fluorescent quantitative PCR

The relative expression levels of iNOS, Arg-1, CD80 and CD206 were determined by reverse transcription fluorescent quantitative PCR with the following primers:
Arg-1: F: 5'-ACCATAGGGATTATTGGAGC-3';
R: 5'-TGTCATTAGGGATGTCAGCA-3';
iNOS: F: 5'-CGTGTTTCACCAGGAGATG-3';
R: 5'-CAGCATACAGGCAAAGAGC-3';
CD80: F: 5'-ACTGGCAAGAATCCAAACCAACCC-3';
R: 5'-GCATTTCTGCAGGTCAGGCATGTT-3';
CD206: F: 5'-GAAATCCCTGCTACTGAACCCC-3';
R: 5'-CAATGGAAACCAGAGAGGAACCC-3';
β-actin: F: 5'-AGCGAGCATCCCCCAAAGTT -3'
R: 5'-GGGCACGAAGGCTCATCATT-3'

Effects of ox-LDL on secretion, phagocytosis and cell survival of macrophages

Cell grouping and drug therapy

After THP-1 cells were induced into macrophages, they were randomly divided into a blank control group, an experimental control group, a drug treatment group.Different concentrations (low dose, ld, 1 μmol/L; Medium dose, md, 10 μmol/L; High-dose, hd, 50 μmol/L) of salbutamol or ICI118551 (different concentrations of metoprolol and propranolol were added in addition to ICI118551 based on known clinical efficacy, for further evidence. Metoprolol doses were the same as before, and the low dose of propranolol was 0.1μmol/L, the medium dose was 1 μmol/L, and the high dose was 10 μmol/L) After 1 hour of pretreatment with each drug, ox-LDL (China Guangzhou Yiyuan Biotechnology Co., Ltd.) with a final concentration of 50 mg/L was added into all groups except for the blank group for incubation for 24 h, and the supernatant and cells were collected.

Changes in the phagocytic ability of macrophages to ox-LDL

The changes in phagocytic ability of macrophages to ox-LDL were observed under oil red staining. The mean optical density and the integrated optical density were then analyzed using Image pro-plus Image Analysis Software (Media Cybernetics).

Cell survival

The cell viability was determined by MTT assay. A total of 100 μ L of ox-LDL-cultured mononuclear cell suspension (1×10^6/L) was placed on sterile plates, and 20 μL of MTT solution was added to each well for incubation for 4 h. The absorbance (OD) of each well was measured at 570 nm.

Enzyme-linked immunosorbent assay

TNFα and IL-10 were determined by ELISA as described above.

Detection of macrophage death mode caused by ox-LDL

Cell grouping and drug therapy

After THP-1 cells were induced into macrophages,

they were randomly divided into a blank control group, an experimental control group, and a drug treatment group. After the different drug treatment groups were pretreated with a medium dose (10 μmol/L) of SAL or ICI118551 for 1 hour, all the groups except for the blank group were incubated with ox-LDL (China Guangzhou Yiyuan Biotechnology Co., Ltd.) with a final concentration of 50 mg/L for 24 h, and the supernatant and cells were collected.

Cell apoptosis and cell membrane rupture death: The cell density was adjusted to 2×10^6/ml, and the cells were incubated with the primary antibody in the dark for 30 minutes after pretreatment with the corresponding drugs, followed by washing with PBS, adding the fluorescently labeled secondary antibody, and culturing in the dark for 30 minutes. The suspension was then reconstituted with 500 liters of PBS and the ratio of apoptotic to non-apoptotic deaths from cell membrane rupture was determined by flow cytometry.

Cell pyroptosis detection

Caspase-1 and GSDMD detected by Western blot

The cells were collected by digestion to extract total protein, which was quantified using the BCA method. According to the protein concentration, 40 μg protein was boiled for denaturation, followed by 12% polyacrylamide gel electrophoresis, and the protein was transferred to the PVDF membrane by wet transfer membrane method. 5% skimmed milk powder (prepared with TBST) was blocked for 1 hour, the primary antibody was diluted in the ratio of 1: 1000 with the primary antibody diluent, and incubated overnight at 4°C. The membrane was shaken and washed three times, 10 minutes for each time, with the addition of the 1: 5000 diluted secondary antibody, which was shaken and incubated for 1 hour at room temperature. After the membrane was shaken and washed three times with TBST, the chemiluminescent solution was added, and the ECL luminescent chemical developing substrate was added according to the kit instruction for overnight exposure developing and protein band observation.

IL-18 and IL-1β were detected by ELISA

Macrophages were inoculated into 96-well plates (1×10^6/ml). Salbutamol and ICI118551 were added to the plate for 1 hour and then 100 μg/ml of ox-LDL was added to the plate for 48 hours. Each group has three composite holes. The culture supernatant was collected after culture, and the levels of IL-18 and IL-1β were detected by ELISA kit.

Detection of lactate dehydrogenase release level

The macrophages were inoculated into 96-well plates (1×10^6/ml). According to the previously set grouping, salbutamol and ICI118551 were added for incubation for 1 hour and then 100ug/ml of ox-LDL was added for incubation for 48 hours. Three re-wells were set for each group, and the background control group and normal control group were also set up. After the culture, reagents were added according to the kit operation steps, and finally the optical density value was detected by a microplate reader with a wavelength of 490nm.

Based on the cytological research results, it is verified whether ox-LDL promotes M2-type differentiation of macrophages and induces the progression of atherosclerosis in *ApoE -/-* mice fed with a high-fat diet in animal models.

Animal programme

Male *ApoE−/−* mice (n=20), aged 5-6 wk, weighing 15±2.2 g, were obtained from ShuLaibao Biotechnology Co., ltd (Wuhan, China). Mice were kept on a 12:12-h light-dark cycle with constant temperature and humidity and food and water freely available. The animal experimental protocol complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and was approved by the Animal Care Committee of Three Gorges University (No.2020010A).

Modeling and grouping

After adaptive feeding for one week, Mice were randomly divided into four groups: control group, model group, SAL group (salbutamol) and ICI group (ICI118 551). The control group received a standard control diet (LAD3001G, containing 20% protein, 70% carbohydrate and 10% fat, purchased from Nantong Trofey Feed Technology Co., Ltd.), and the other groups were fed a high-fat diet (Van Heek series 60% high-fat obesity model feed TP23300, containing 20% protein, 20% carbohydrate and 60% fat, purchased from Nantong Trofey Feed Technology Co., Ltd.) for 12 weeks to prepare a mouse atherosclerosis model (21).

Medication

The SAL group was administered 50 mg / 1000 ml of salbutamol (MCE, USA) in drinking water. The ICI group was administered 15 mg / 1000 ml of the selective B2-adrenergic receptor antagonist ICI 118551 in its drinking water (MCE, USA) (22). After the last dose, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium, the eyeball was removed, and blood was collected from the post-orbital hemorrhage. The serum was centrifuged at 4°C. The levels of total cholesterol (T-CHOL), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured by an automatic hematology analyzer. The contents of TNF and IL10 in blood were determined by ELISA.

Histological and morphological analyses

Mice were anesthetized by intraperitoneal pentobarbital injection and perfused through the left ventricle with with PBS. The whole aorta was separated and the remaining fat and connective tissue were removed, followed by washing with PBS and fixation in 4% paraformaldehyde for more than 12 h.

The heart and aorta were dissected, and fixed in 4% paraformaldehyde for 48 hours, The heart and aorta were dissected and fixed in 4% paraformaldehyde for 48 hours. Some samples were longitudinally sectioned from the root of the main artery to the iliac artery, with the tail bifurcation also sectioned. After being rinsed with PBS and 60% isopropyl alcohol, oil red staining was performed, and pictures were taken after drying in a good white light. Image-Pro Plus 6.0 (Media Cybernetics) was used to measure the plaque area, the area of the fibrous cap and lipid core, and the thickness of the fibrous cap, and the area ratio of the fibrous cap to lipid core was obtained. To calculate the plaque destruction rate, a given animal was counted only once, regardless of how many sites in the specimen had

plaque destruction.

The rest aorta was excised from below the atrial appendage, dehydrated with sucrose, and embedded in an OCT complex. For rapid freezing, 50 consecutive sections with a thickness of 8 μm were sectioned at 100 μm intervals from the aortic valve using a cryomicrotome. The sections were stained with hematoxylin and eosin. Serial cryosections were cut at 8 μm thickness every 100 μm along the aorta artery specimens, and 50 sections per vessel were obtained. The sections were stained with hematoxylin and eosin. The largest site of the plaque was selected for morphological analysis. The plaque area, the area of the fibrous cap and lipid core as well and the thickness of the fibrous cap were measured using Image-Pro Plus 6.0 (Media Cybernetics), and the area ratio of the fibrous cap to core was obtained. To calculate the plaque destruction rate, no matter how many parts of the specimen showed plaque destruction, a given animal was counted only once.

The remaining sections were subjected to immunofluorescence analysis with the following antibodies: F4/80, CD86, and CD163. After being photographed under a fluorescence microscope, the number and distribution of F4/80-positive, CD86-positive and CD163-positive macrophages were qualitatively analyzed using Indica labs (USA) software, and the tri-positive F4/80+CD86+CD163 or dual-positive co-expression of CD86+CD163 was identified through image stacking (23). The number of positive cells and the total number of cells in the target area were quantified using Halo v3.0.311.314 (USA) image analysis software in order to calculate the positive rate (%).

Statistical Analysis

Data are expressed as mean±standard deviation (SD). The analysis of variance was used for comparison between groups. The statistical design was ANOVA and multiple comparisons were analyzed using the least significant difference (LSD) back testing. and A level of $P<0.05$ was considered statistically significant. All analyses involved the use of Statistic Package for Social Science (SPSS) 23.0 (SPSS, IBM, Armonk, NY, USA).

Results

Macrophages were successfully prepared

The THP-1 cells were in good condition under the light microscope, with round or oval shapes, large size and clear nuclei. Some cells gathered to form clusters and were in a suspended state. After 24 h of PMA induction, THP-1 cells began to adhere to the wall, differentiate, and show prominent pseudopodia, suggesting that the induction of macrophages was successful.

Effects of β2-AR agonists or β2-AR blockers on the differentiation of macrophages into M1 or M2 types

LPS+IFN-γ induced M1 transformation of THP-1-derived macrophages and IL4-induced M2 transformation

After induction of THP-1-derived macrophages with LPS+IFN-γ, compared with the control group, the expression levels of TNF-α (Figure 1A), iNOS (Figure 1C), and macrophage surface CD80 (Figure 1E) were increased, suggesting M1-type differentiation of macrophage. After induction of THP-1-derived macrophages with IL-4, compared with the control group, the expression of IL-10 (Figure 1B), Arg-1 (Figure 1D), and macrophage surface CD206 (Figure 1F) were increased, suggesting M2-type differentiation of macrophage.

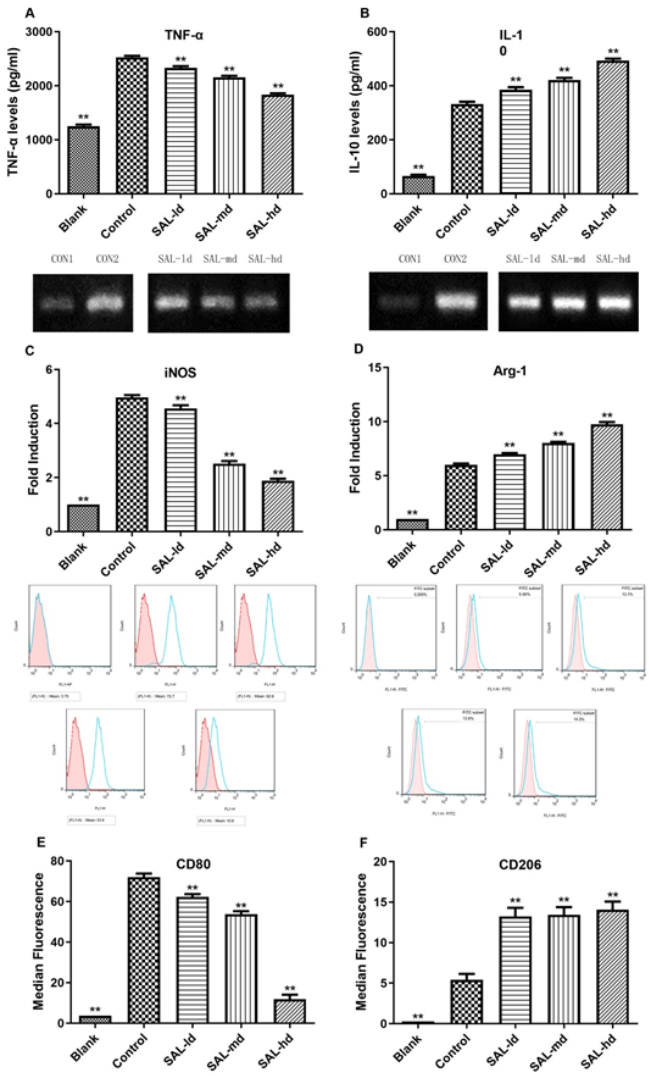

Figure 1. Salbutamol inhibited the polarization tendency of macrophage into the M1 type induced by LPS+IFN-γ and promoted the polarization tendency of macrophage into the M2 type induced by IL-4. (A, C and E) showed the levels of TNFα (ELISA), iNOS (PCR) and CD80 (FCM) in macrophages induced by LPS+IFN-γ, respectively. (B, D and F) showed the levels of IL-10 (ELISA), Arg-1 (PCR) and CD206 (FCM) in macrophages induced by LPS+IFN-γ, respectively. SAL: Salbutamol; Ld: low dose; Md: medium dose, hd: high dose; **: $P<0.05$ compared with Control group; n=5.

β2-AR agonist inhibits the M1 type differentiation of macrophages and promotes the M2 type differentiation of macrophages

In LPS+IFN-γ-induced macrophages, salbutamol significantly reduced TNF-α expression (Figure 1A), iNOS expression (Figure 1C), andCD80 expression on the macrophage surface in a concentration-dependent manner (Figure 1E). The above results suggested that β2-AR agonist inhibited the differentiation of macrophages into M1 type. In IL-4-induced macrophages, salbutamol significantly increased IL-10 secretion (Figure 1B), Arg-1 expression (Figure 1D), and CD206 expression on the macrophage surface in a concentration-dependent manner (Figure 1F). The above results suggested that β2-AR ago-

nist promoted the M2-type differentiation of macrophages.

β2-AR blockers promote the M1 type differentiation of macrophages and inhibit the M2 type differentiation of macrophages

In LPS+IFN-γ-induced macrophages, ICI118551 (as well as metoprolol or propranolol) significantly increased TNF-α expression (Figure 2A, Figure 3A, Figure 4A), iNOS expression (Figure 2C, Figure 3C, Figure 4C) and CD80 expression on macrophage surface (Figure 2E, Figure 3E, Figure 4E) in a concentration-dependent manner, suggesting that β2-AR blocker promoted macrophage differentiation into M1 type. In IL-4-induced macrophages, ICI118551 (as well as metoprolol or propranolol) significantly reduced the expression of IL-10 (Figure 2B, Figure 3B, Figure 4B), Arg-1 (Figure 2D, Figure 3D, Figure 4D) and CD206 on the macrophage surface (Figure 2F, Figure 3F, Figure 4F) in a concentration-dependent manner, suggesting that β2-AR blocking agent inhibited the M2-type differentiation of macrophages.

The results of the above studies indicated that β2-AR agonists and blockers could be used as intervention drugs for subsequent experiments.

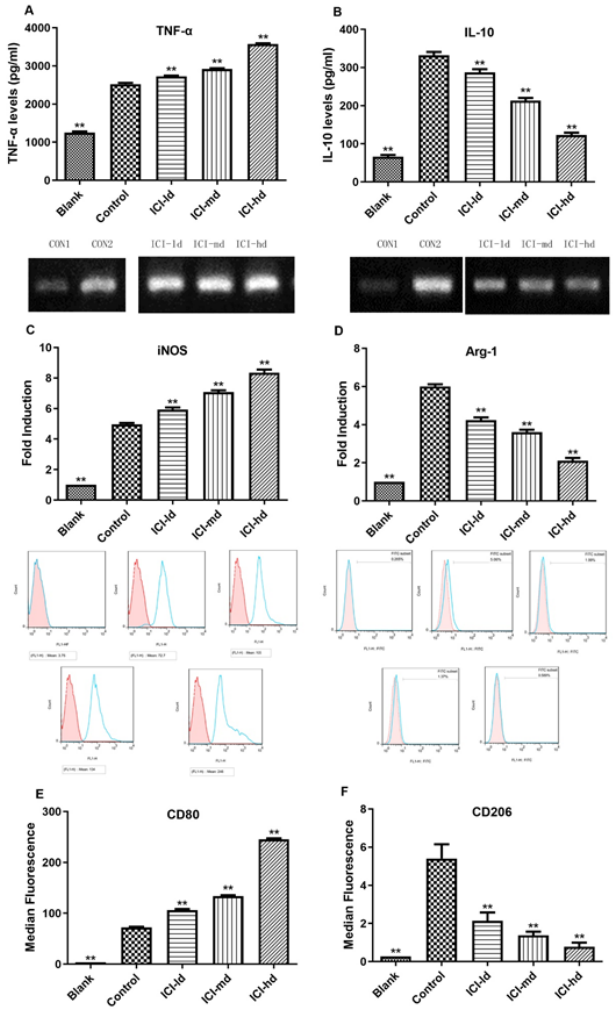

Figure 2. ICI118551 inhibited the polarization tendency of macrophage into the M2 type induced by IL-4 and promoted the polarization tendency of macrophage into the M1 type induced by LPS+IFN-γ. (A, C and E) showed the levels of TNFα (ELISA), iNOS (PCR) and CD80 (FCM) in macrophages induced by LPS+IFN-γ, respectively. (B, D and F) showed the levels of IL-10 (ELISA), Arg-1 (PCR) and CD206 (FCM) in macrophages induced by LPS+IFN-γ, respectively. ICI: ICI118551; Ld: low dose; Md: medium dose, hd: high dose; **: P<0.05 compared with Control group; n=5.

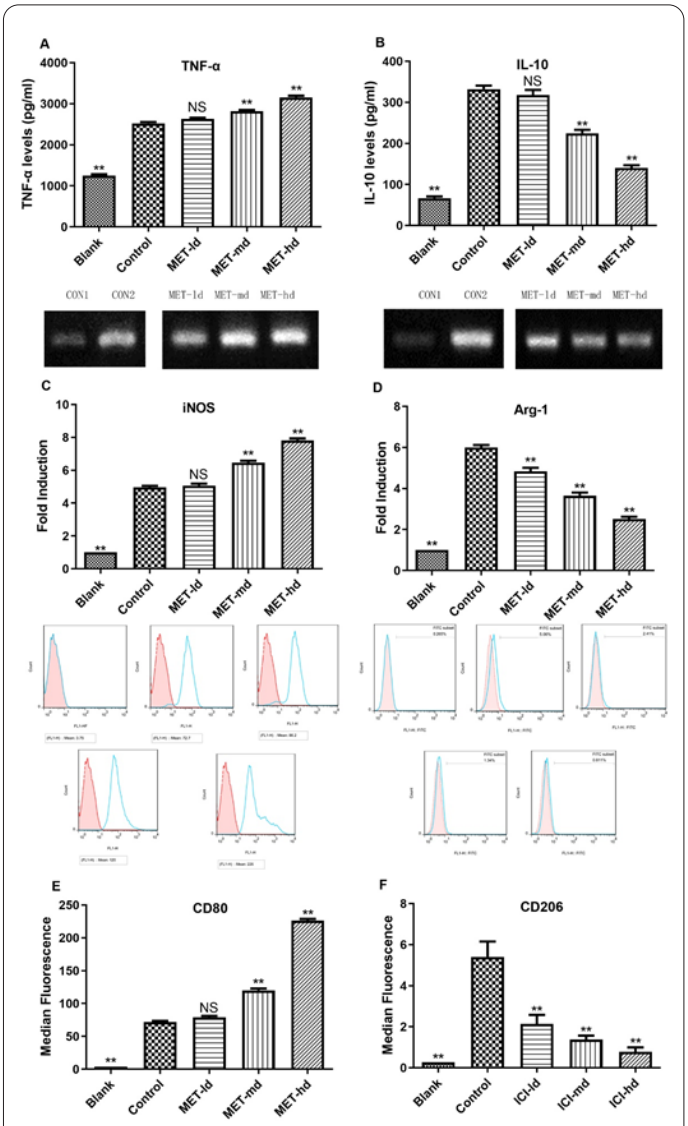

Figure 3. Metoprolol inhibits IL-4-induced polarization of macrophage M2 and promotes LPS+IFN-γ-induced polarization of macrophage M1. A: LPS+IFN-γ-induced secretion of macrophage TNFα (ELISA), B: IL-4-induced secretion of macrophage IL4 (ELISA), C: LPS+IFN-γ-induced expression of macrophage iNOS (PCR), D: IL-4-induced expression of macrophage Arg-1 (PCR), E: LPS+IFN-γ-induced expression of macrophage CD80 (FCM), F: IL-4-induced expression of macrophage CD206 (FCM). MET: metoprolol; Ld: low dose; Md: medium dose, hd: high dose; * *: p<0.05, n=5 compared with the Control group

Ox-LDL promotes the M2-type differentiation of THP-1-derived macrophages and impacts the function of macrophages, death and pyroptosis pathways

THP-1-derived macrophages were incubated with ox-LDL for 24 hours. Under oil red O staining, THP-1-derived macrophages incubated with ox-LDL contained a large number of bright red lipid particles, and foam cells were formed, suggesting that the atherosclerotic cell model was successfully established.

Ox-LDL promotes M2 type differentiation of macrophages

Compared with the control group, ox-LDL reduced the secretion of TNF-α of macrophages, increased the secretion of IL-10, decreased the expression of iNOS and

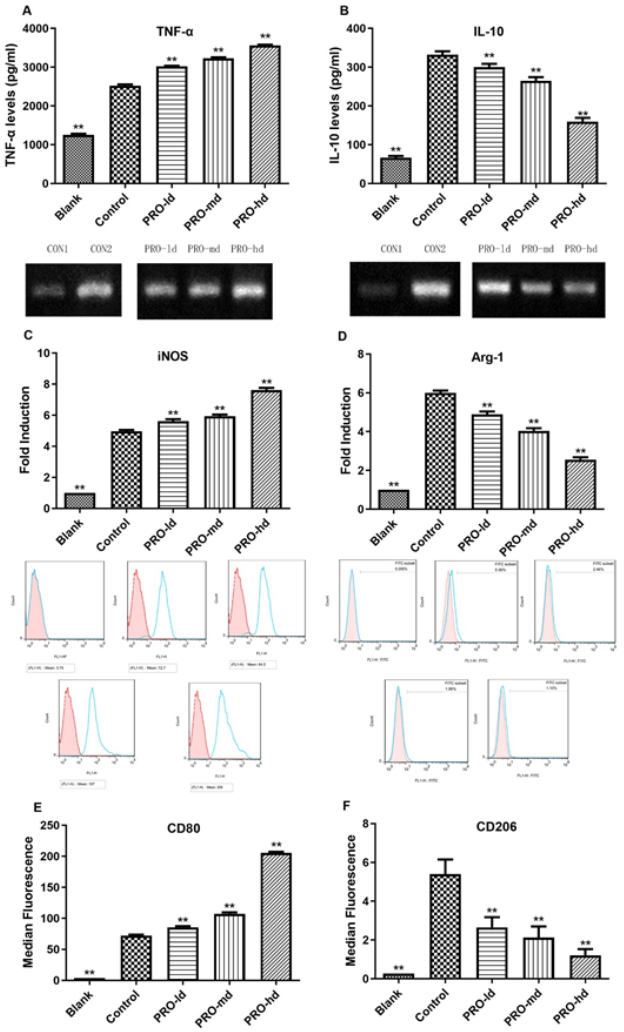

Figure 4. Propranolol inhibits IL-4-induced polarization of macrophage M2 and promotes LPS+IFN-γ-induced polarization of macrophage M1 A: LPS+IFN-γ-induced secretion of macrophage TNFα (ELISA), B: IL-4-induced secretion of macrophage IL4 (ELISA), C: LPS+IFN-γ-induced expression of macrophage iNOS (PCR), D: IL-4-induced expression of macrophage Arg-1 (PCR), E: LPS+IFN-γ-induced expression of macrophage CD80 (FCM), F: IL-4-induced expression of macrophage CD206 (FCM). PRO: propranolol; Ld: low dose; Md: medium dose, hd: high dose; * *: p<0.05, n=5 compared with the Control group.

CD80, and increased the expression of Arg-1 and CD206. This effect was further enhanced by salbutamol and attenuated by ICI118551 (Figure 5).

Ox-LDL enhances the phagocytic function of macrophages, reduces the survival rate, reduces the secretion of TNF-α, and increases the secretion of IL-10

Compared with the control group, the phagocytosis of ox-LDL by macrophages in the ox-LDL group was significantly increased the survival rate of macrophages was significantly decreased, the secretion of TNF-α was decreased, and the secretion of IL-10 was increased (Figure 6).

β2-AR agonists enhance the effects of ox-LDL on macrophage function

Compared with the ox-LDL group, Salbutamol (1 μmol/L) had no significant effects on the phagocytic function, survival rate and TNF-α and IL-10 secretion of macrophages However, Salbutamol (10 μmol/L and 50 μmol/L) increased the phagocytic effect of macrophages on ox-LDL in a concentration-dependent manner, significantly reduced the survival rate of macrophages in a concentration-dependent manner, and significantly reduced TNF-α secretion by macrophages and increased IL-10 secretion in a concentration-dependent manner (Figure 6).

β2-AR Blockers inhibit the effect of ox-LDL on macrophage function

Compared with the ox-LDL group, ICI118551 at different concentrations could reduce ox-LDL phagocytosis of macrophages in a concentration-dependent manner. Different concentrations of ICI118551 could increase the survival rate of macrophages in a concentration-dependent manner. ICI 118551 at different concentrations increased the secretion of TNF-α and decreased the secretion of IL-10 in a concentration-dependent manner. Metoprolol (MET) and propranolol (PRO) with different β2-AR blocking effects had the same effect (Figure 6)

Ox-LDL promotes apoptosis and non-apoptotic death of macrophages

The rates of macrophage apoptosis and cell membrane rupture mortality were significantly higher in the ox-LDL

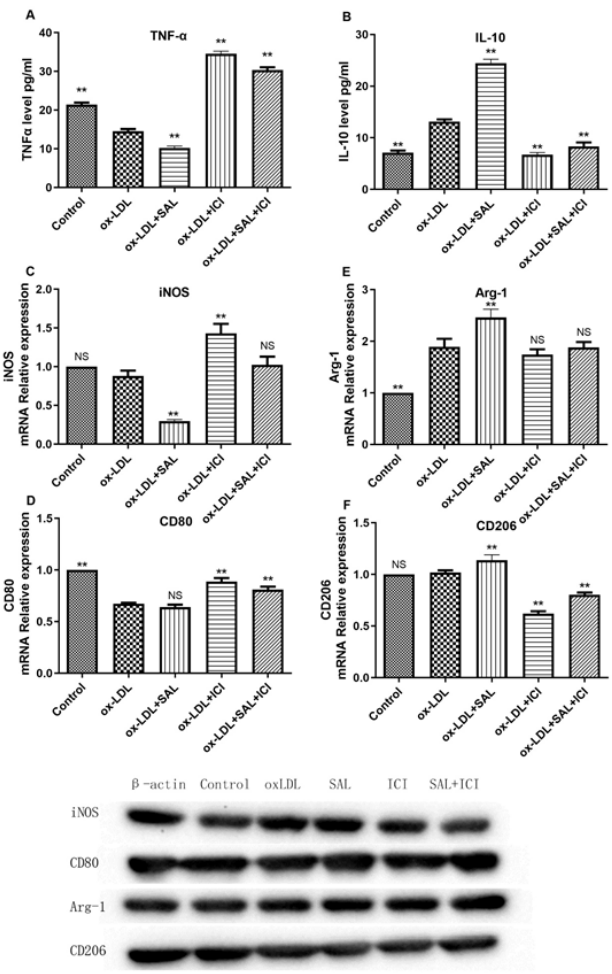

Figure 5. ox-LDL promotes the polarization differentiation of macrophages into M2 type, salbutamol promotes it and ICI118551 inhibits it. (A-F) showed the effects of different drugs on the levels of TNFα (ELISA), IL-10 (ELISA), iNOS (PCR), Arg-1 (PCR), CD80 (PCR) and CD206(PCR) in macrophages induced by ox-LDL. SAL: salbutamol; ICI: ICI118551; **: Compared with ox-LDL group, P < 0.05; NS: compared with ox-LDL group, P>0.05; n=5.

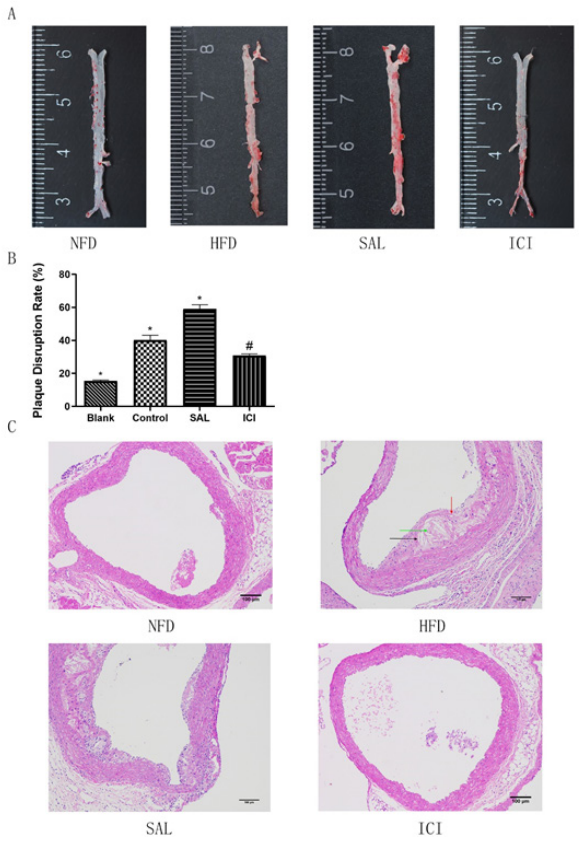

Figure 6. The phagocytic function of macrophages incubated with ox-LDL is enhanced, and the cell survival rate is decreased. The secretion of IL-10 is increased, while that of TNFα is decreased, which is promoted by salbutamol and inhibited by ICI118551, metoprolol or propranolol. A-D sequentially showed the changes of average optical density after macrophages swallowed ox-LDL under oil red O staining. E-H in sequence showed the change in macrophage survival rate detected by the MTT method. I-L in sequence showed the changes in TNFα secretion level detected by ELISA. M-P sequentially showed the changes in IL-10 secretion level detected by ELISA. SAL: salbutamol; ICI: ICI118551;MET: metoprolol; PRO: propranolol; ld: low dose; md: medium dose, hd: high dose; * *: P < 0.05 compared with the Control group; NS: p>0.05, n=3 compared with the Control group.

group compared with the control group. Salbutamol was increased and ICI118551 weakened this effect (Figure 7).

Ox-LDL induced pyroptosis of macrophages

The results showed that compared with the control group, the expressions of caspase-1 and GSDMD proteins in the ox-LDL group were increased, and the secretion levels of IL-18 and IL-1β were significantly increased. Optical density values after LDH release in each group: Compared with the control group, LDH release was increased in the ox-LDL group. Salbutamol promoted ox-LDL-induced autophagy and ICI118551 inhibited ox-LDL-induced autophagy (Figure 7).

In animal models, ox-LDL promotes M2-type differentiation of macrophages and induces the progression of atherosclerosis that occurs in *ApoE* -/- mice fed a high-fat diet

Male *ApoE* -/- mice aged 5–6 weeks were divided into different groups. After the model group and the medication group were fed with a high-fat diet for 12 weeks, the mouse atherosclerosis model was prepared. The biometric measurement indicated that the serum total cholesterol, low-density lipoprotein cholesterol, and triglyceride levels were increased (Table 1). HE or oil red O staining of vascular sections at the aortic root showed that atherosclerotic plaques developed well (Figure 8A, 8C), indicating that the model was successfully established.

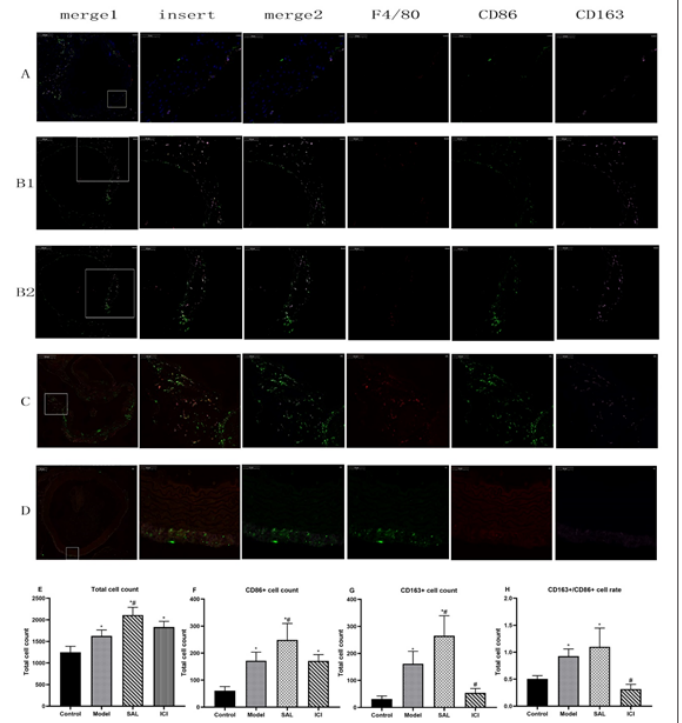

Figure 7. Increased apoptotic and non-apoptotic deaths of macrophages incubated with ox-LDL, with activation of the focal apoptotic pathway and the death of cell membrane rupture, promoted by salbutamol and inhibited by ICI. The apoptotic rate (A) and necrotic rate (B) of macrophages were detected by flow cytometry. The expression levels of Procaspase-1(C), caspase-1(D) and GSDMD protein (e) in each group were detected by Western Blot. The optical density (F) of LDH after release was detected by ELISA reader. The levels of IL-18(G) and IL-1β(H) in each group were detected by ELISA. SAL: salbutamol, ICI: ICI11855; *, P<0.05, n=3 compared to the ox-LDL group.

Table 1. Basic characteristics and biochemical parameters in four groups of mice.

Parameters	control (n=5)	model (n=5)	SAL (n=5)	ICI (n=5)
Body weight, g	26.95±0.93	26.70±2.00	27.02±0.85	27.07±2.03
Total cholesterol, mmol/L	5.08±0.50	6.73±0.53*	6.72±0.39*	7.06±0.45*
LDL cholesterol, mmol/L	4.42±0.37	5.04±0.47*	5.01±0.44*	5.28±0.33*
HDL cholesterol, mmol/L	1.50±0.24	1.51±0.13	1.59±0.16	1.77±0.14
Triglyceride, mmol/L	0.34±0.02	0.51±0.09*	0.54±0.09*	0.54±0.08*

Value are the means±SD for n animals. LDL, low-density lipoprotein; HDL, high-density lipoprotein; SAL: salbutamol, ICI: ICI118551; *P<0.05 compared with Control group, #P<0.01 compared with Control group, n=6.

Body Weight

There was no difference in body weights between the four groups.

Biological Measurements

Serum total cholesterol, LDL cholesterol, and triglyceride levels in the Model, SAL, and ICI groups were significantly higher than those in the control group, and there were no significant differences among the three groups. There was no significant difference in serum HDL cholesterol among all groups.

Oil red O stain in longitudinal sections of the aortic root

After 12 weeks, the mice were dissected, and the longitudinal sections of the aortic roots were stained with oil red O. A typical sample is shown in Figure 8A: The fatty plaque in the vascular wall is bright red, and other parts are nearly colorless. Quantitative analysis of typical samples showed that compared with the control group, the fat plaque areas in the model group and SAL group were increased by 51.9% and 61.7%, respectively, and that in the ICI group were increased by 25.6%. The plaque destruction rates in the control group and ICI group were lower than that in the model group, and that in the SAL group was higher than that in the model group. Moreover, there were significant differences in plaque destruction rate between each group and the model group. The plaque destruction rate in the ICI group was higher than that in the control group and there was a significant difference (Figure 8B).

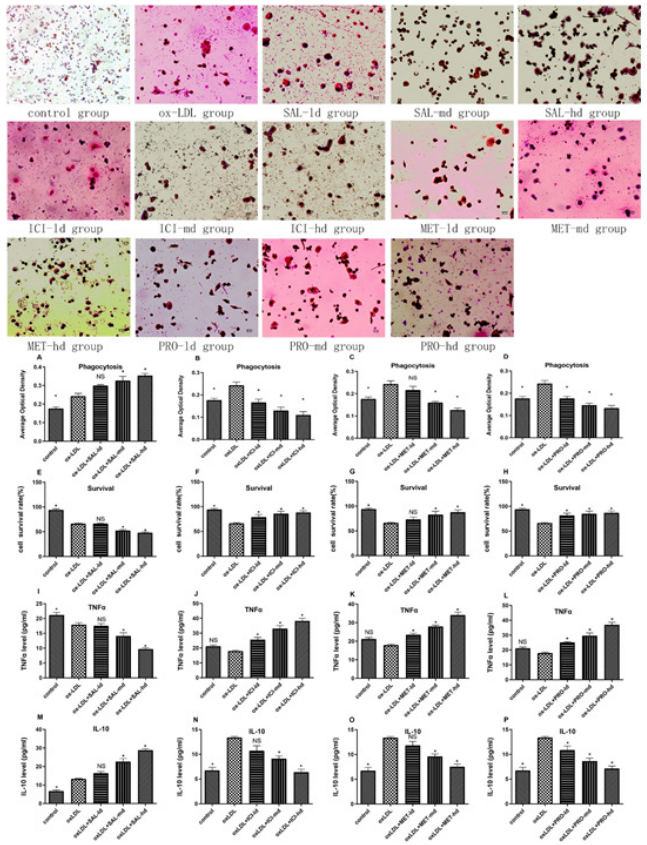

Figure 8. Progress of atherosclerosis in ApoE-/- mice fed with high-fat diet promoted by albuterol, and delayed by ICI118551. A shows the longitudinal section of the aorta stained with oil red O, B shows the comparison of plaque destruction rates among different groups, and C shows the transverse section of the aortic root stained with HE. The black arrows indicated cholesterol crystals, the red arrows indicated foam cells and the green arrows indicated atherosclerosis and necrosis. SAL: salbutamol, ICI: ICI11855; * compared with the model group, $P < 0.05$, # compared with the control group, $P < 0.05$, n=6.

Pathological manifestation of atherosclerosis in each group by HE staining

After 12 weeks of the high-fat diet, the atherosclerotic plaques in the model group developed well. In contrast, no atherosclerotic plaques were found in the control group after 12 weeks of regular diet. Figure 8C shows the phenotypic characteristics of the plaque. Morphological analysis showed that the inner and outer layers of the vascular wall in the control group were normal. A large number of atherosclerotic plaques, cholesterol crystals and foam cells were observed in the model group. The SAL group had more foam cells, disintegrants and cholesterol crystals, while ICI group had normal blood vessels and some foam cells.

Expression of the number and distribution of aortic macrophages in each group by immunofluorescence staining

The effect of β2-AR agonists or antagonists on the expression levels of surface markers associated with the polarization state of diseased macrophages was detected by immunofluorescence staining (Figure 9A-D). Representative images show that several CD86-positive (M1) macrophages are visible in the endovascular membrane of ApoE -/- mice (control group) fed with ordinary diet for 12 weeks, and CD163-positive cells (M2) are visible in the adventitia. However, the total number is not large, and no double-positive co-localization of CD86 and CD163 is observed. In the model group, a lipid-striated plaque and an AS-developing plaque were found in the same section. The number of M1-type and M2-type macrophages in the lipid-striated plaque was increased. M1-type macrophages were mainly distributed at the intima of the vascular lumen, with the shoulders on both sides of the lipid-striated section obvious. M2-type macrophages were mainly distributed at the proximal, middle and outer membrane inside the lipid-striated. On the partial lipid-striated side of the M1-type macrophage population in the lipid-striated shoulder, multiple CD86 and CD163 double-yang co-localization were observed, and two CD86 and CD163 double-yang co-localization were observed inside the lipid-striations. In the developing plaque of the Model group, the numbers of M1-type and M2-type macrophages were significantly increased. M1-type macrophages were also mainly distributed at the vascular intima, especially in the shoulders on both sides of the cut surface of the lipid plaque. M2-type macrophages were mainly distributed near the vascular medial and adventitia in the lipid plaque. In the developing plaques, more double-positive co-localization of CD86 and CD163 was observed in the M1 type macrophage population located on the partial plaque side of the shoulder, and a small number of double-positive co-localization was observed in the plaques. In the SAL group, multiple AS lesions and a large, more developed plaque were found in the same section. A large number of M1-type macrophages were observed in the shoulder of the plaque, and a large number of M1-type and M2-type macrophages were observed in the plaque. A large number of CD86 and CD163 double-positive co-localizations were observed in

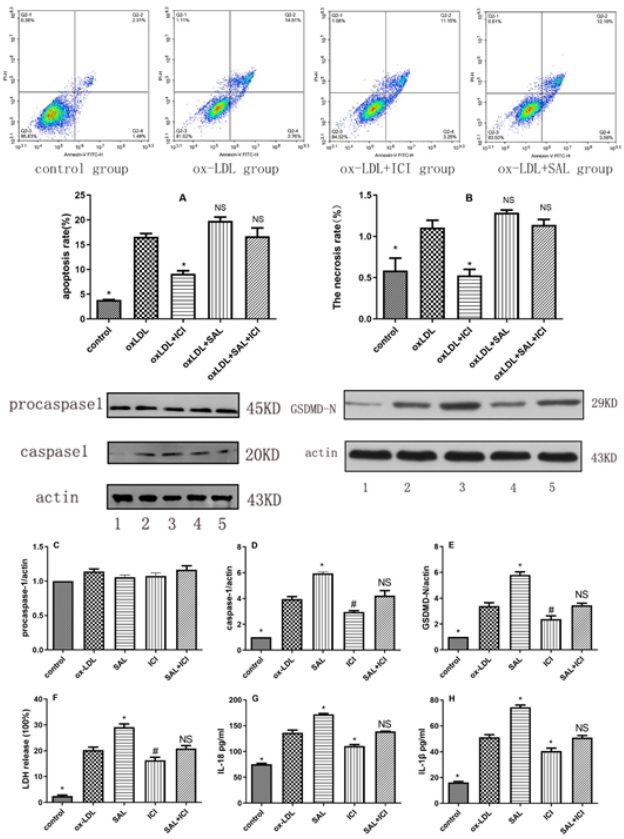

Figure 9. Immunofluorescence staining of transverse section of aortic root. Immunofluorescence staining was performed on transverse sections of aortic roots. A: control group, B1: model group 1 (with enlarged lipid striations), B2: model group 2 (with enlarged plaques), C: SAL group, and D: ICI group. Macrophages were identified as F4/80+ (red) cells, M1 macrophages as CD86+ (green) cells, and M2 macrophages as CD163+ (pink) cells, recognizing the co-expression of F4/80 with CD86 and/or CD163 by image overlay. merge1: F4/80+CD86+CD163 stained image overlay, inset: The white box in merge1 is enlarged, merge2: Overlay of CD86+CD163 stained images; The target M1 and M2 macrophages in each section were quantitatively analyzed to count the total cell count (E), CD86+ cell count (F), and CD163+ cell count (G). SAL: salbutamol, ICI: ICI118551; * Compared with the control group, $P < 0.05$, # compared with model group 1, $P < 0.05$, n=6.

the M1-type macrophage population located on the side of the plaque and in the plaque at the shoulder of the plaque. No CD86-or CD163-positive stained cells were observed in the intima of the aortic root in the ICI group, and no CD86-positive macrophages and no CD163-positive stained cells were observed in the adventitia.

Quantitative analysis of macrophage in target regions M1 and M2 of each slice (Figure 9E-G) show:

Total number of macrophages

Compared with the control group, the total number of macrophages in other groups was higher than that in the control group. Compared with model group 1 (lipid streak), model group 2 (plaque) and SAL group were higher than model group 1 and both had significant differences. There was no significant difference in the total number of cell 1 between the ICI group and the model group.

Positive rate of CD86+ macrophage (M1)

Compared with the control group, the positive rates of CD86+ macrophages in other groups were significantly increased. Compared with model group 1, the positive rates of CD86+ macrophages in model group 2, SAL group and ICI group were significantly increased, and SAL group was the highest.

CD163+ Macrophage (M2) Positive Rate

Compared with the control group, CD163+ macrophages in the control group were significantly lower than those in model group 1, model group 2, and the SAL group, and there was no significant difference between the two groups. There was no significant difference in CD163+ macrophages between model group 1 and model group 2, but they were all significantly lower than the SAL group, and higher than the control group and the ICI group.

Discussion

In this study, firstly, through cytological experiment, we verified that β2-AR, β2-AR stimulant on the surface of macrophages can not only promote the conversion of macrophages to M2 type but also inhibit the conversion of macrophages to M1 type (24), and confirmed that β-AR blockers with different degrees of blocking effect on β2-AR, including metoprolol, propranolol and ICI118551, are agonists that can regulate the polarization state of macrophages in the opposite way to that of β2-AR stimulant salbutamol through β2-AR (25-27). The β2-AR stimulant or the β-AR blocker performs mutual reverse intervention of promotion or inhibition on the process that ox-LDL promotes the M2 type differentiation of macrophages, further verifying that ox-LDL can promote the M2 type differentiation of macrophages (18-20), and further verifies that M2 type macrophages are more sensitive to lipid toxicity than M1-type macrophages (19,28). However, its phagocytic ability is stronger than that of M1-type macrophages (7), and it is more likely to form foam cells (19,29). M2-type macrophages that engulf ox-LDL are more likely to die than other forms of macrophages (19,28). The in-depth study on the mode of death of macrophages after consuming ox-LDL has revealed that, in addition to apoptosis, non-apoptotic modes of death such as pyroptosis, in which the cell membrane is ruptured, are also included. Therefore, the cytological finding that ox-LDL promotes M2-type differentiation of macrophages offers the possibility of disrupting the procedural order that M1-type and M2-type macrophages must follow in the early stage of inflammation-repair/regeneration response in AS. Macrophage death is the key cause of lipid necrosis core formation in AS plaques (30-33). M2-type macrophages easily die after consuming lipids, which also provides the possibility of promoting the formation of lipid necrosis core in AS plaques and leading to the progression of AS.

To explore whether ox-LDL can cause persistent progression of AS by disrupting the procedural order that M1-type and M2-type macrophages must strictly follow in vivo during the inflammatory response phase, our group used the cytological research results to guide the animal experimental research. Pathological analysis of vascular specimens obtained from animal experiments showed that the AS lesions and plaque destruction rates observed in the control group, model group and SAL group were more serious according to the implemented enhancement of the action intensity of AS factor. The distribution of M1-type

macrophages in AS plaques in each group showed that the intima of the control group was slightly damaged, while M1-type macrophages only appeared at the intima of the blood vessel. The M1-type macrophages in fatty streak plaques of the model group appeared under the intima and at the shoulder. The M1 type macrophages in developing plaques appeared under the intima and the shoulder was significantly increased. For the more serious plaques in the SAL group, the M1 type macrophages were more significantly visible from the intima to the plaque and were characterized by a significant increase in the shoulder. Similar findings have been reported in multiple studies (34,35). The above histopathological features suggested that in the AS animal model established with *ApoE -/-* mice, during the formation of AS plaques, there was a phenomenon that monocytes penetrated the intima of the vascular lumen to develop into M1-type macrophages and were recruited with the lipid injury area as the center. The "shoulder" of plaque is the place where M1 type macrophages tend to concentrate in the lipid injury area, i.e., they will enter the lipid injury area and contact with lipids. In vivo studies (36,37) have found that when monocytes recruit into AS plaques to form foam cells, the pro-inflammatory properties of macrophages are unexpectedly lost. Flow cytometry analysis of foam cell subsets of diseased macrophages revealed that approximately 22% of these cells exhibited "M2" or "selectively activated" macrophage characteristics. The above studies suggested the existence of interconversion of M1 type and M2 type macrophages in the formation of AS plaques in the body. Studies have found the presence of M1 and M2-labeled doubly positive co-located macrophages in the lesions of myocardial infarction. After a series of experiments, it is considered that the reason is the differentiation of M1-type macrophages into M2-type (2). In addition, in the mouse AS plaque regression model, M1 and M2 double positive marker co-located macrophages were also found, suggesting the existence of transformation of M1 type macrophages to M2 type (38). In another study, foam cells containing M1 and M2 markers were found in the M1 type macrophage population at the shoulder of the human unstable AS plaque specimen, but no evidence was found to support the M1 type differentiation of M2 type macrophages (35). In this study, the image superposition method (23) was adopted, and it was also found that there was double positive co-localization of M1 and M2 markers in AS plaques. The distribution characteristics of these double positive and co-localization phenomena were as follows: The vessels in the control group produced the least damaging response. the double-positive co-localization phenomena were only distributed at the intima of blood vessels in the model group and the SAL group; with the increase of the action intensity of the factor causing AS, the lesion degree of AS plaques worsened, and successively presented the trend of development from under the intima to the deeper level of plaques, with the number gradually increasing; the overall distribution trend was completely consistent with the distribution trend of M1 type macrophages. Especially in the "shoulder" with more M1-type macrophages in AS plaques, the double-positive co-localization phenomenon was more common in the direction toward lipid plaques of M1-type macrophages, which was closely related to the lipid plaques. It has been found in multiple cytological studies, including this one, that ox-LDL can promote M2-type differentiation of macrophages (18-20). In conclusion, the double positive co-localization of M1 and M2 markers found in AS lesions in this study highly suggests that recruited M1-type macrophages can be converted to M2-type by lipid induction in the early stage of inflammation-regeneration/repair reaction after contact with lipids. If it is considered that the double-yang co-localization is formed by the M1 type differentiation of M2 type macrophages after consuming lipids, the distribution of the double-yang co-localization should not be consistent with the distribution trend of M1 type macrophages infiltrating into AS plaques, and it should be significant in the shoulders with lipid striations (where macrophages contact lipids), but in the center of lipid striations in the early lesions of AS, because a large number of M2 type macrophages that engulf lipids have already existed in the lipid striations. According to the cytological experimental results, it is speculated in this research group that the pro-inflammatory effect exhibited by M2-type macrophages after lipid phagocytosis is more likely related to the release of IL-1β and other substances after the activation of the focal death pathway, and is not caused by its transformation into M1-type macrophages. Although double-positive macrophages of Arg I and Arg II have been found in the vasculopathy of *ApoE -/-* mice fed with a normal diet for 55 weeks (33), since this phenomenon was not found in the vasculopathy of *ApoE -/-* mice fed with normal diet for 20 weeks, the researchers believe that double positive macrophages of Arg I and Arg II are caused by the active inflammatory environment inducing macrophages to transform from M2 to M1 in the late stage of AS plaque formation. The related literature (33) found that in the provided picture of *ApoE -/-* mice fed with the ordinary diet for 55 weeks, the AS lesion was atypical and should still belong to the earlier AS lesion. Literature images showed that double-positive macrophages of Arg I and Arg II were located at the margin of the AS lesion in the M1 type macrophage population under the intima. In the opinion of this research group, we cannot rule out that the double positive macrophages of Arg I and Arg II observed in the literature may still evolve from recruited circulating mononuclear cells after they are converted into M1-type macrophages. In this study, we considered that the M2-type differentiation of recruited M1-type macrophages after lipid contact had significant pathological significance for AS plaque formation: in the stage of as inflammation, ox-LDL should be treated by M1-type macrophages which have better lipid treatment ability than M2-type (7) and are not easy to form foam cells (34). However, under the induction of ox-LDL, the recruited M1-type macrophages differentiate into M2-type, which is determined by the anti-inflammatory properties of M2-type macrophages. Depending on whether the disease is alleviated, M2-type macrophages have poor treatment ability for ox-LDL, which is not conducive to the harmless treatment of ox-LDL, and it is easy to form foam cells with the functional property of M2-type (34). M2-type macrophages have poor tolerance to ox-LDL and are prone to non-apoptotic death (such AS pyroptosis) including cell membrane rupture, which has become a hidden danger for the further sustainable development of AS. When the foam cells die, the ruptured cell membrane undergoes non-apoptotic death (such as pyroptosis) to release damage-related molecular patterns (DAMPS). The circulating monocytes continue to be recruited into the endo-

metrium and activated into M1-type macrophages, which are then induced by ox-LDL to differentiate into M2-type cells, which then engulf and die. After repeated circulation, the AS inflammation course presents chronic manifestations. Studies using the bead-tracking method have found that latex beads in AS plaques can be transferred from phagocytes to phagocytes (35), confirming the existence of this adverse cycle.

Through observing the relationship between the number of M2-type macrophages and the formation of AS plaques, we also found that the number of M2-type macrophages under the intima of rats in the model group was significantly higher than that in the control group. The TNF-α in peripheral blood was significantly lower than that in the control group, and IL-10 was significantly higher than that in the control group. The plaque destruction rate was higher than that in the control group, and AS plaques were formed. The number of M2-type macrophages in the SAL group was significantly higher than that in the model group. The TNF-α in the peripheral blood was significantly lower than that in the model group, and IL-10 was significantly higher than that in the model group. The plaque destruction rate was significantly higher than that in the model group, and more severe AS plaques were formed. Clinical investigation has also revealed that for the population with AS lesions, patients with high IL-10 levels have poor prognosis (40). The macrophages of *ApoE -/-* mice themselves have a significant M2-type differentiation tendency (23). When *ApoE -/-* mice are fed a high-fat diet, ox-LDL in AS plaques increases, and it is easy to induce the M2-type differentiation and even proliferation of macrophages in plaques (7,41). The number of M2-type macrophages or the polarization degree of M2-type macrophages is increased, so that the lipid-phagocytosis is more active. More macrophages consume lipids and then undergo non-apoptotic death (such as pyroptosis), releasing injury-related molecular patterns, and recruiting M1-type macrophages to promote the progression of AS.

If non-apoptotic death (such AS focal death) occurs due to excessive M2-type macrophages in AS plaques, a large number of M1-type macrophages will converge on the shoulder of AS plaques due to chemotaxis, and even cause shoulder rupture of AS plaques due to the pro-inflammatory action of M1-type macrophages themselves. Endoplasmic reticulum stress has been found to promote the M2-type differentiation of macrophages and the progression of AS plaques, and it has been proposed to promote the M1-type differentiation of M2 macrophages and prevent the formation of AS plaques (29). Pathological studies on the inflammatory response induced by tissue injury have revealed that the number of tissue macrophages contained in the tissue before tissue injury is positively correlated with the intensity of inflammatory response after tissue injury. Reducing the number of tissue macrophages (12) or consuming specific cell subsets (14) at an early stage usually reduces the related inflammatory response. β-receptor antagonists with β2-AR selectivity can reduce the reactivity of white blood cells to injury and alleviate tissue damage (41). In this study, cytological experiments showed that β-AR blockers metoprolol, propranolol and ICI118551, which had different degrees of selectivity for β2-AR, had the effects of promoting the differentiation of macrophages into M1 type and inhibiting the differentiation into M2 type.

In animal experiments, the number of M2-type macrophages under the intima of mice in the ICI group was only equivalent to that in the control group. The TNF-α in peripheral blood was significantly higher than that in the model group, and IL-10 was significantly lower than that in the model group, which was also equivalent to that in the control group. Although the plaque destruction rate in the ICI group was higher than that in the control group, it was significantly lower than that in the model group, and there was no AS plaque formation, suggesting that there was an adverse correlation between M2-type differentiation of macrophages and AS plaque formation. Therefore, reducing the number of M2-type macrophages in plaques or the degree of M2-type polarization and preventing non-apoptotic death (such AS focal death) including cell membrane rupture induced by excessive lipid phagocytosis of M2-type macrophages may be the important mechanism of the above-mentioned β-AR blocker inhibiting the progression of AS. Previous studies (42) and other studies (40-42) of this research group showed that β-AR blockers with different degrees of blocking effect on β2-AR: including metoprolol (43,44), propranolol (44-46), carvedilol (47) and nebivolol (48,49) had significant anti-AS effects. Therefore, reducing the number of M2-type macrophages in plaques or the degree of M2-type polarization and preventing non-apoptotic death (such AS pyroptosis) including cell membrane rupture induced by excessive lipid phagocytosis of M2-type macrophages may be the important mechanism of the above-mentioned β-AR blocker inhibiting the progression of AS. Evidence-based medicine research has found that especially long-term use of β-AR blockers can slow the development of AS (50-52), which has inadvertently provided a forerunner for the conclusion of this study.

Limitations

Due to the difficulty in tracking living cells, we have not conducted a strict and clear tracking study on the participation of macrophages in the AS formation process. Only the cytological research conclusion can be used as a clue to guide animal experimental research. According to the distribution characteristics of vascular M1 type and M2 type macrophages and M1 type and M2 type labeled double positive co-localization in mouse aortic root by immunofluorescence staining and its relationship with lipid plaques, it is analyzed and judged that the double positive co-localization of M1 type and M2 type markers is formed by the M2 type differentiation of M1 type macrophages into AS after they contact with lipids.

The lipid toxicity of ox-LDL causes tissue damage under the intima. As ox-LDL induces the recruited monocytes to differentiate into M2-type macrophages, M2-type macrophages first handle the pathogenic factors in the early stage of inflammation-repair/regeneration reaction, disrupting the procedural rules that M1-type and M2-type macrophages must follow. M2-type macrophages are prone to death (such as pyroptosis) including macrophage membrane rupture, so that circulating monocytes are constantly recruited, and AS inflammation is rendered chronic. Ox-LDL induces the number of M2-type macrophages in plaques to be increased or the M2-type polarization degree to be increased, and more M2-type macrophages die after consuming lipids, thereby enhancing the intensity of inflammatory response in atherosclerotic

plaques, promoting the progression of atherosclerosis, and reducing the number of M2-type macrophages in plaques or the M2-type polarization degree to slow the progression of atherosclerosis. The conclusions of this study have provided a new understanding of the pathological mechanism of AS and a new perspective for targeted therapy of AS in clinical practice.

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Disclosures
None.

Author contributions
Jingqun Zhou conceived and designed the experiments. Bo Zhang, Weichang Xia, Xijian Liu, Xuan Du, Rong Tu and Shouyi Liu performed the experiments and prepared the manuscript. Jingqun Zhou and Bo Zhang participated in discussions of data analysis. Jingqun Zhou revised the manuscript. All the authors gave final approval.

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