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ORIGINAL ARTICLE

Synergistic effect of scavenger receptor A and low-density lipoprotein receptor on foam cell formation

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Abstract: The aim of the study is to investigate the effect of LPS on the expression of scavenger receptor A (SR-A) and low-density lipoprotein receptor (LDL-R) genes and proteins in RAW264.7 cell line. RAW264.7 cells were incubated in serum-free medium randomly in the absence or presence of low-density lipoprotein (LDL) alone, LDL+LPS and LPS alone. Intracellular cholesterol contents were assessed by Oil Red O staining cholesterol enzymatic assay, tumor necrosis factor α levels in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA), mRNA and protein expressions of SR-A and LDL-R in the treated cells were assessed by semi-quantitative polymerase chain reaction and western blot, respectively. LPS was able to up regulate SR-A mRNA and protein expressions, override LDL-R suppression induced by a high dose of LDL and increase LDL uptake by enhancing receptor expression, leading to foam cell formation in Raw264.7 cells. Synergistic effect of the upregulation of SR-A and dysregulation of LDL-R under inflammatory stress may contribute to macrophage-derived foam cell formation.

Keywords: Scavenger receptor A; Low-density lipoprotein receptor; RAW264.7 macrophages; Atherosclerosis; Inflammation.

Introduction

Atherosclerosis is characterized by lipid (mainly for cholesterol and cholesterol ester) accumulation and infiltration of inflammatory cells (especially macrophages)[1]. Accumulation of lipoprotein in the arterial wall is one of the important cause of atherosclerosis[2-3]. In blood vessel walls, low density lipoprotein (low density lipoprotein, LDL) is prone to be captured in the matrix proteoglycan oxidative modification, causing local inflammation leading to mononuclear macrophages collection in the arterial wall[4]. Macrophages ingest modified LDL, results in the formation of foam cells, a process that is characteristic of early atherosclerotic lesions[5].

As a form of coronary artery disease, vascular inflammation is the main cause of atherosclerosis, at the same time also has the very serious clinical manifestations[6]. Inflammation is usually associated with higher levels of inflammatory markers, and is closely related to inflammatory stress. At the same time, chronic inflammation also can increase the risk of cardiovascular disease[7,8]. However, most of atherosclerosis and inflammatory stress mechanism remains to be studied. Inflammation and infection may affect the level of plasma cholesterol and lipoprotein by

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regulating the synthesis and secretion of apolipoprotein, lipolysis enzyme activity, or the expression of lipoprotein receptor. Two main determinant of plasma cholesterol levels is used to adjust the macrophages intake modified LDL of scavenger receptor A (scavenger receptor A, SR-A) and regulation of natural LDL intake of low density lipoprotein receptor (low density lipoprotein receptor, LDL-R)[9–10]. By oxidation of LDL to stimulate macrophage and SR-A expression in cells. At the same time, the SR-A also closely associated with oxidation of LDL[9–11]. However, macrophages LDL-R and SR-A roles are still not very clear in inflammation. This study aims to explore the LPS influence on macrophage accumulation of cholesterol, and high dose of LDL to LPS stimulation of macrophage RAW264.7 cell line of LDL-R and SR-A gene and protein expression level of influence.

Materials and methods

Cell culture

Macrophage RAW264.7 cell line with contain RPMI-1640 medium, 10% fetal bovine serum, 2 mmol/L glutamate, 100 U/ml penicillin and 100 ug/ml streptomycin medium. All experiments in containing 0.2% bovine serum albumin, 2 mmol/L glutamate, 100 u/ml and 100 ug/ml penicillin streptomycin serum-free medium RPMI-1640. Cell culture reagents and LPS were from Sigma company. LDL was the continuous ultracentrifugation obtained from normal human plasma.

Enzyme-linked immunosorbent assay (ELISA) to detect TNF-α

Cultivation of RAW264.7 cells under 37℃ with PBS cleaning was performed twice, followed by the addition of 1 ml fresh, serum-free medium RPMI-1640. After 24 h, 1 ml containing or not containing 100 ng/ml LPS medium was added. The corresponding time was needed for cell incubation experiment according to ELISA kit (eBioscience, UK) specifications, determination of the concentration of TNF α in cell supernatant.

The determination of cholesterol in the cell

According to description of Gallo and Gamble[17–18], enzymatic detection was used to detect cholesterol levels in the cell. Six of RAW264.7 cells in the orifice plate was divided into the control group, the LDL group (100 ug/ml), the LPS group (100 ng/ml) and LDL LPS group, and cultivated 24 h, then the cells with PBS cleaning was performed twice, followed by isopropyl alcohol lipids extraction in the cell, and drying in vacuum. Enzymatic detection was used to determine the contents of total cholesterol, free cholesterol, cholesterol ester as the difference between total cholesterol and free cholesterol. Then, total protein cells were determined by Lowry test to standardize the results.

Oil red O staining

RAW264.7 cell cultured in serum-free medium for 24 h, and then were given different stimulus. After 24 h, cells with 5% formalin fixed 30 min at room temperature, soaking in oil red O staining solution at room temperature 30 min, washed three times, and then wood staining was used to display the nucleus.

The total RNA extraction and RT-PCR

Trizol reagent (Promega, USA) was used to check on the total RNA from the cultured cells. Reverse transcription from Toyoba kit (500 ng) total RNA was used as a template for reverse transcription. Reverse transcription reaction containing 50 1KCl, 10 tendency for l/l Tris HCl, 5 tendency MgCl2, 1 tendency for each type of deoxyribonucleic acid

(DNA), 2.5 umol/l random primers, 20 u/l RNA enzyme inhibitors and 50u Moloney mouse leukemia virus mixture, was thermocycled at room temperature in DNA Thermal Cycler PCR (Eppendorf, USA) followed by incubation in 10 min, then in 42 ℃ for 30 min incubation and incubated with the 99 ℃ for 5 min. PCR reaction mixture from DongSheng Biotec Co., China was used in DNA Thermal Cycler PCR (Eppendorf, USA) in semi-quantitative PCR. Reaction conditions as follows: 95 ℃ for 10 min, 30 95 ℃ for 30 s circulation, 51℃, 30 s, 72 s 45 ℃. Electrophoretic separation of PCR product, Genegenus system and version 3.05 GeneTools software (Synoptics, UK) was performed to calculate relative content of mRNA. The house kept genes of GAPDH used as reference. Oligonucleotide primers sequence:

SR-A Upstream 5`-TCAATGACAGCATCCCTTCC-3`

Downstream 5`-ATGTCCTCCTGTTGCTTTGC-3`;

LDL-R Upstream 5`-TTGCAGTAGAAGACTCAGGC-3`,

Downstream 5`-ATGATTTGCAGCGGAAGTGG-3`;

GAPDH Upstream 5`-ATTCAACGGCACAGTCAA-3`,

Downstream 3`-TGAGGGTGAGAAGGTGGAA-5`.

Protein imprinting analysis

Under 4 ℃, 150 cells was cultured in bottle with 300 ul cell lysis liquid (10 tendency lHEPES, pH 7.9, 10 tendency lKCl, 1.5 tendency lMgCl2, dithiothreitol tendency for 0.5 l, 0.4 tendency Nonidet P/l-40,0.5 umol/l benzyl sulfonyl fluoride, 1 ug/ml resistance protease, 1 ug/ml light antimicrobial peptide enzyme, 1 ug/ml inhibin butyl benzene, 1 ug/ml or curd protease element) pyrolysis, 23 GA needle through 20 times, and then 4 ℃, 14000 g centrifugal 15 min. Protein was extracted from the cells, supernatant. Equal to the total Protein denaturation heating, in the Bio-Rad Mini Protein instrument in 5% concentration on the glue, 8% separating gel electrophoresis. Turn 60 v, 200 ma film 2 h, DianZhuan liquid is 25 tendency Tris, Ph 8.3, tendency for 192 / l glycine, 0.1% SDS and 20% methanol. With 5% skim milk closed 1 h at room temperature, PBST membrane washing twice, 5 min each time. 1% bovine serum albumin in PBST antibodies as diluent, dilution after A fight (sheep source SR-A antibody and rabbit source LDL antibodies, rabbit source SREBP-2-R, purchased from Santa Cruz, USA) 2 h incubation, two resistance (2 fight for the sheep, the sheep of rabbit rabbit, purchased from Santa Cruz, USA) in antibody diluent dilution, film in two fight in 37 ℃ for 1 h incubation, PBST cleaning three times, each time for 5 min. To join the ECL luminescent reagents, in Genegnome system (Synoptics) exposure, the use Quantity One software banding (Bio-Rad, UK) analysis.

Immunocytochemistry staining

RAW264.7 cells with 5% formalin fixed at room temperature 30 min, washing three times, according to with immunohistochemical reagent instructions of BOSHIDE of Wuhan City, Rabbit anti rabbit LDL-R antibody was used for first antibody, PBS was used as a negative control.

Statistical methods

The data test, t between groups, all analysis were conducted using SPSS 18.0 software, $P < 0.05$ was indicated as significant differences.

Results

ELISA to detect the expression of TNF alpha

The LPS stimulation in RAW264.7 cells for the production of TNF α whether time dependence, and whether LPS cause inflammatory stress were explored. LPS stimulation RAW264.7 cells generated by the level of TNF α on 4 h began to rise, 16 h peaked and continued to 48 h.

Cholesterol enzymatic detection the accumulation of cholesterol in the cell

The LPS effects on cholesterol content in RAW264.7 cells were studied. According to the total cholesterol in the cell and the result of cholesterol ester (*Figure 1A*), total cholesterol and inflammation with RAW264.7 cells concentrations of cholesterol ester and the formation of foam cells.

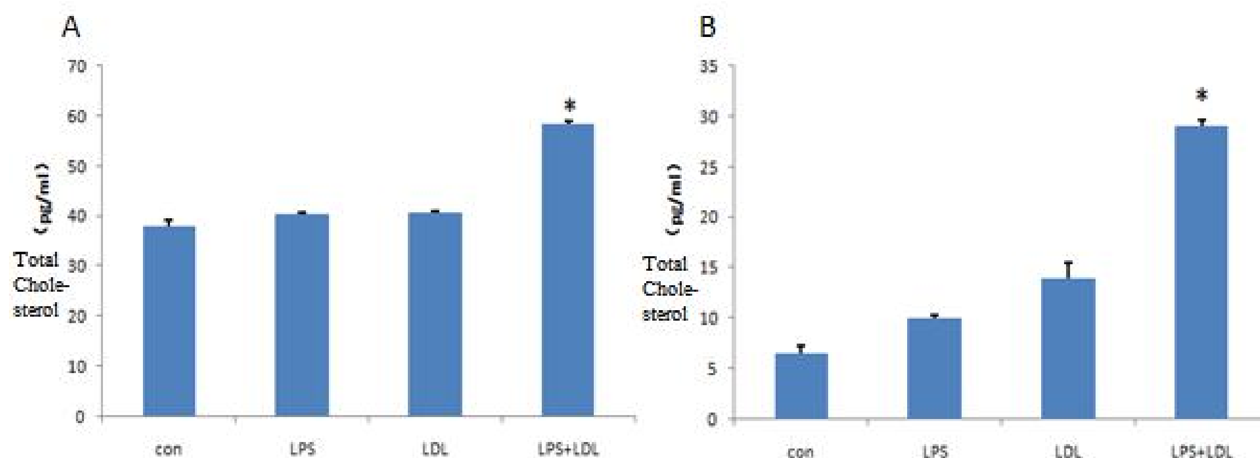

Figure 1. The influence of LPS on cholesterol RAW264.7 cell aggregation. Cells (5x106 / ml) cultured with serum free medium for 24 h, discard supernatant, join the serum-free medium RPMI-1640 respectively 100 ug/ml LDL (control group), medium (LDL), medium 100 ng/ml LPS group (LPS), medium 100 ug/ml LDL 100 ng/ml LPS (LDL LPS group) 24 h. Collect cells for total cholesterol test (A) cholesterol ester test (B). After cell total protein for standardization, and three independent experiment repeated. By t test, found that compared with LPS group, LDL and LPS group have significant difference ($P < 0.05$ (two-way)

Oil red O staining to detect cholesterol accumulation in the cell

Compared with control group, 100 ug/ml LDL processing of RAW264.7 cells and 100 ng/ml LPS treatment all showed a slight increase in the number of lipid droplets (*Figure 2A-C*). 100 ug/ml LDL and 100 ng/ml LPS to stimulate the cells show more significant accumulation of cholesterol (*Figure 2D*). These results are consistent with cholesterol enzymatic results.

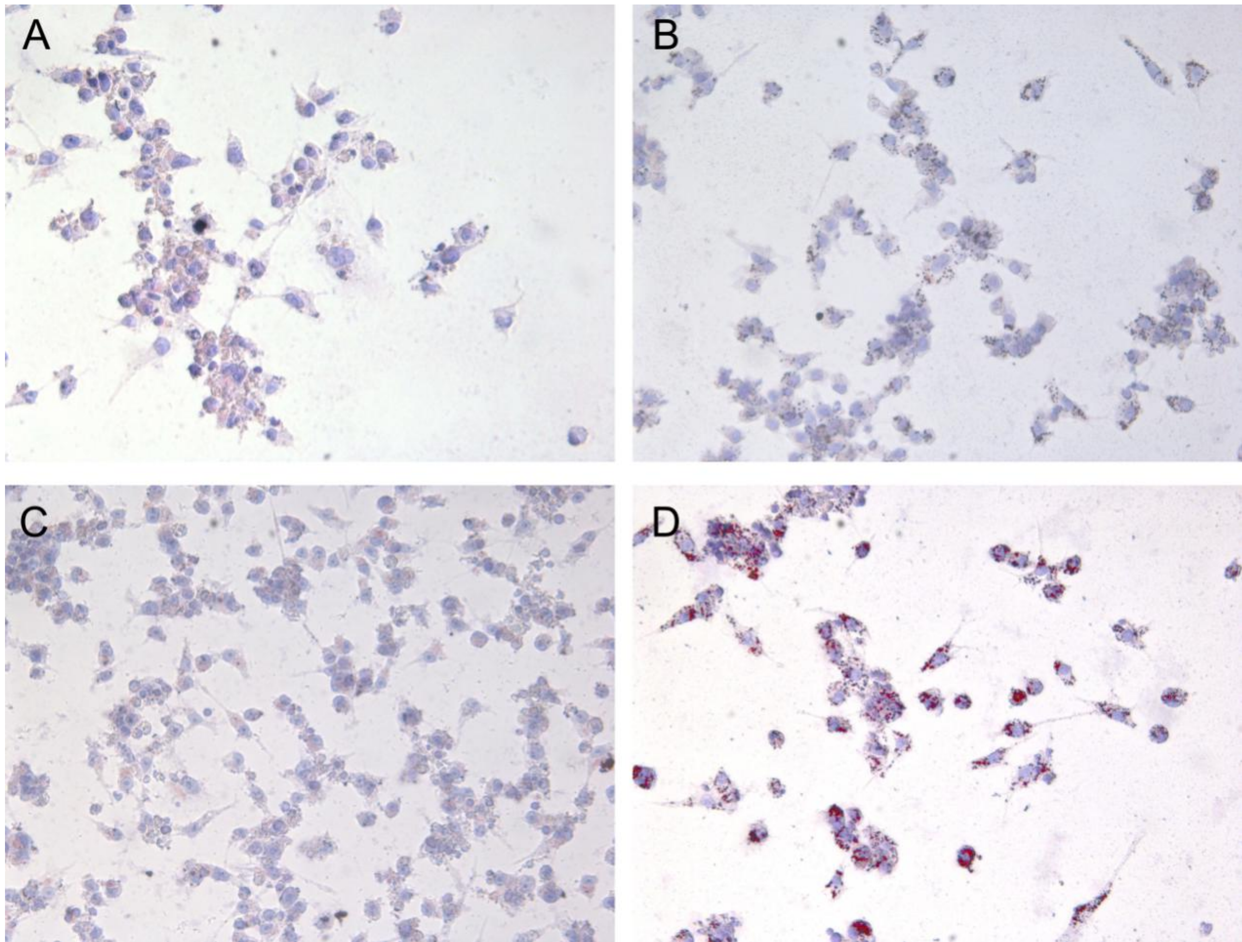

Figure 2. Oil red O staining showed that the accumulation of cholesterol in the cell (SP 10 x 40). RAW264.7 cell culture in serum-free medium for 24 h. Discard supernatant, respectively give serum-free medium RPMI-1640 (the control group A), medium 100 ug/ml LDL (LDL groups, B), medium 100 ng/ml LPS (LPS group, C), medium 100 ug/ml LDL 100 ng/ml LPS (LDL LPS group, D). After 24 h, the cells with 5% formalin fixed 30 min at room temperature, room temperature in the oil red O staining solution soak for 30 min, washing three times, and then use wood staining to display the nucleus.

Expression of mRNA of RT-PCR detection of LDL-R and SR-A

To explore the mechanism of intracellular cholesterol increases caused by LPS induced, RT-PCR detection of RAW264.7 cells of LDL-R and mRNA expression of SR-A. RAW264.7 cells separately in 100 ug/ml LDL, 100 ng/ml LPS, 100 ug/ml LDL 100 ng/ml LPS stimulation for 24 h. Semi-quantitative RT-PCR method to detect mRNA expression. LDL and LPS can increase the SR RAW264.7 cells-A mRNA expression (*Figure 3A*). LDL in-hibiting LDL-R mRNA expression and LPS can interfere with the results, cause LDL-R mRNA expression in RAW264.7 cells (*Figure 3B*)

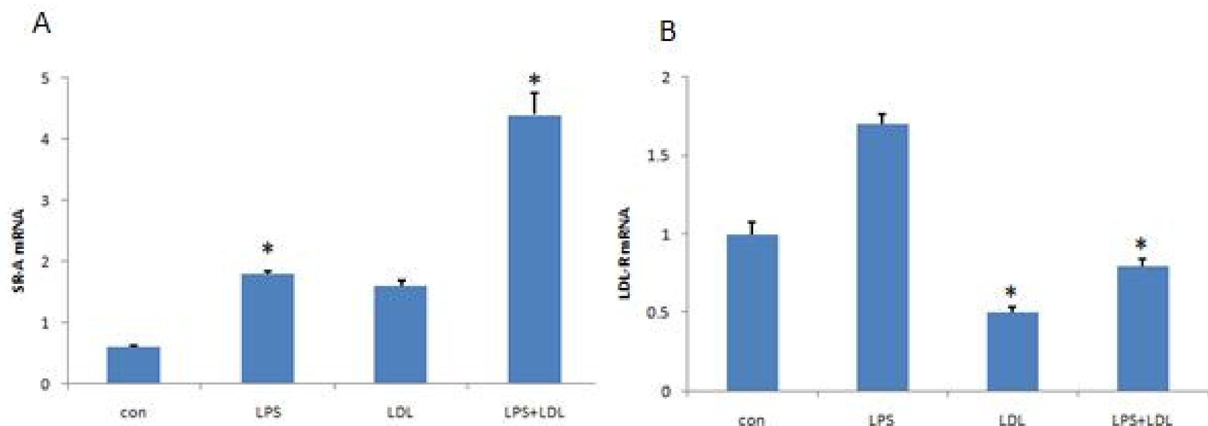

Figure 3. RT-PCR detection of RAW264.7 cells SR-A and mRNA expression in LDL-R. Then, give the serum-free medium RPMI-1640 respectively 100 ug/ml LDL (control group), medium (LDL), medium 100 ng/ml LPS group (LPS), medium 100 ug/ml LDL 100 ng/ml LPS (LDL LPS group). RNA preparation and rt-pcr operations are carried out in accordance with the materials and methods described in. The target length: The SR-A, 550 bp. The experimental results were repeated three times. Compared with control group, the t test found that tag group have significant difference ($P < 0.05$). Compared with LDL group, the t test found that LDL and LPS group have significant difference ($P < 0.05$)

Protein imprinting method to detect LDL_R and SR-A protein expression

Protein imprinting results show that the LDL or LPS can increase the SR RAW264.7 cells, a protein expression ($P < 0.05$) (*Figure 4*). LDL group compared with control group, LDL can inhibit LDL-R RAW264.7 cell protein expression ($P < 0.05$). These results were consistent with the results of the mRNA.

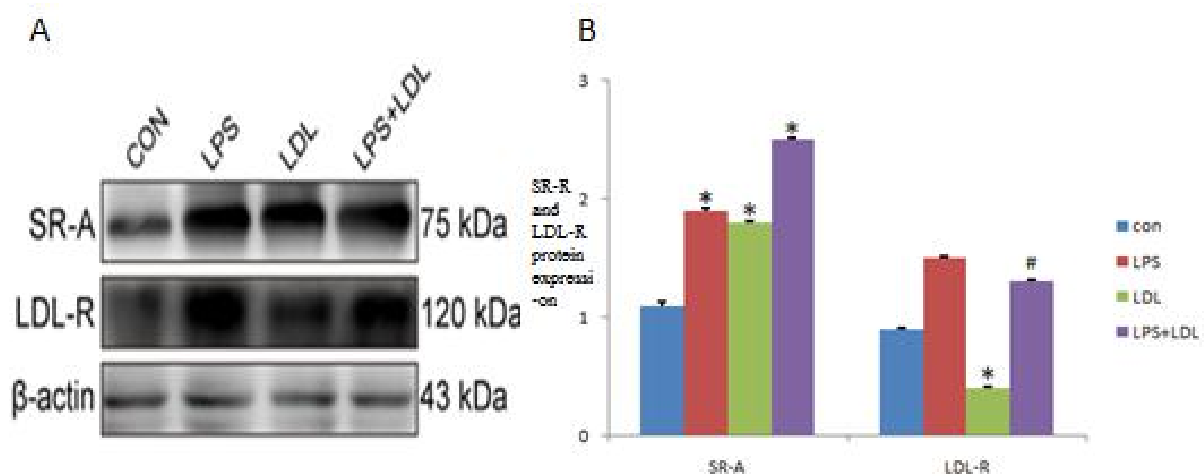

Figure 4. Protein imprinting method to detect the SR RAW264.7 cells-A (A) and LDL-R (B) protein expression. Cells in serum-free culture medium for 24 h, then, were given serum-free medium RPMI-1640 100 ug/ml LDL (control group), medium (LDL), medium 100 ng/ml LPS group (LPS), medium 100 ug/ml LDL 100 ng/ml LPS (LDL LPS group). Tag group results compared with control group, the t test with significant difference ($P < 0.05$, two-way test). Compared with LDL group, the t test found that LDL and LPS group have significant difference ($P < 0.05$, two-way

test).

Immunocytochemistry staining detection of LDL-R expression in RAW264.7 cells

Immunocytochemistry staining showed LDL inhibits the protein expression of LDL-R, and LPS reversible inhibition of LDL (*Figure 5*).

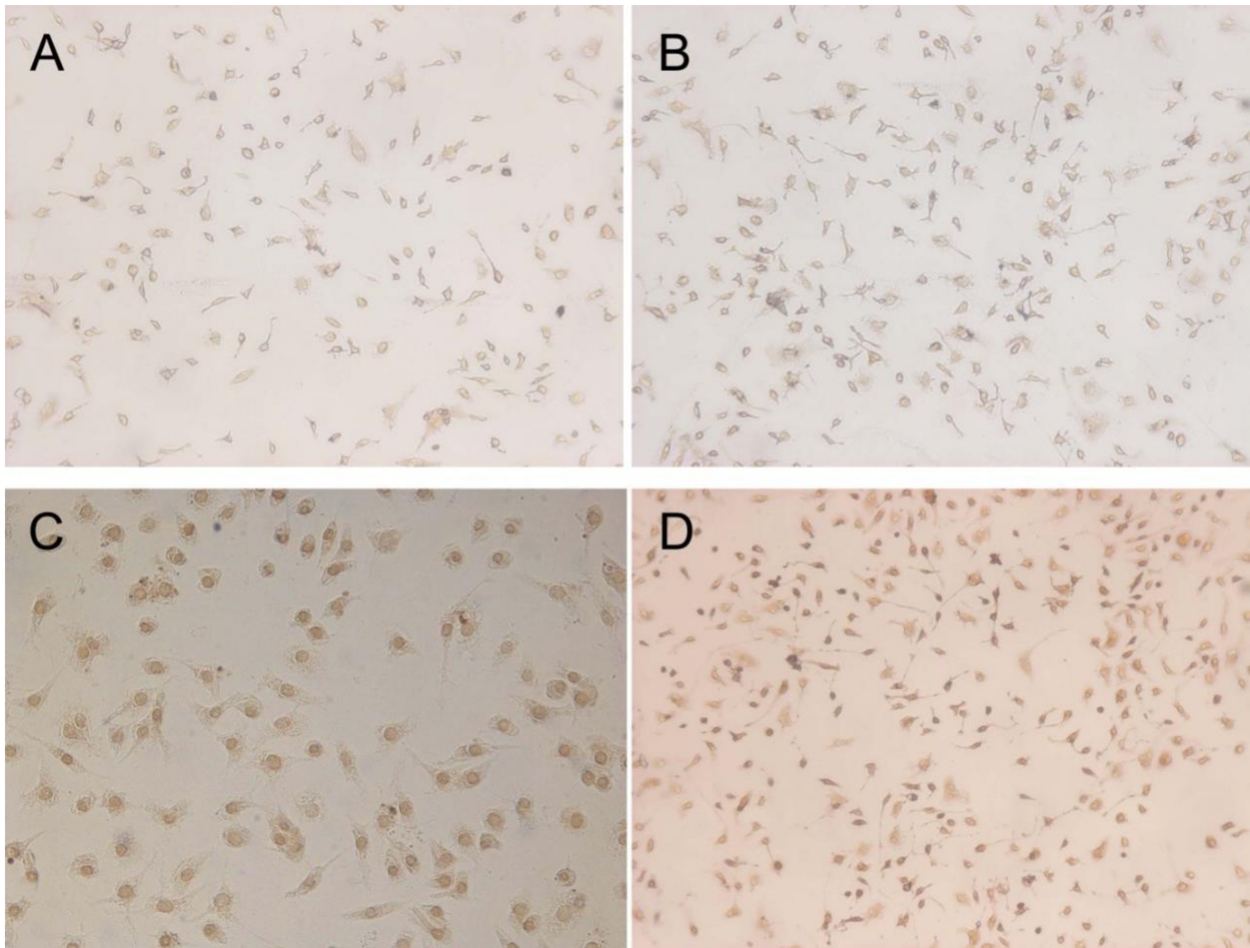

Figure 5. Immunocytochemistry staining showed that protein levels of LDL-R (SP 10 x 20). Cells in serum-free culture medium for 24 h, then, were given serum-free medium RPMI-1640 100 ug/ml LDL (control group), medium (LDL), medium 100 ng/ml LPS group (LPS), medium 100 ug/ml LDL 100 ng/ml LPS (LDL LPS group). After 24 h, the cells with 5% formalin fixed 30 min at room temperature, then according to the reagent instruction immunocytochemistry staining. Finally, use wood staining showed nuclei.

Discussion

In the process of atherosclerosis, the formation of foam cells has long been regarded as the important factor in the development of damage[5,13], participation in lipid intake of scavenger receptor is closely related to the pathogenesis of the disease[9]. Macrophages are considered to be one of the causes of the formation of foam cells[5,12]. Macrophages have many physiological functions, including the secretion of growth factors and cytokines, the removal of the particles in the blood, and so on. Macrophages play an important role in atherosclerosis. In the process of atherosclerosis, inflammation can regulate the expression of receptors on the surface of some of the macrophage, which affect

cholesterol intake and the formation of foam cells[14]. SR-A is an important part of the cell surface receptors, can be combined with A modified LDL[9]. LDL-R can be combined with plasma source sex LDL cholesterol, which regulates blood plasma LDL levels[10]. Some studies have reported the SR-A and LDL-R on macrophage derived foam cell formation of single function (15–16th). However, the synergy of both studies was very few. In this study, we mainly discuss the LPS induced inflammation under the condition of the receptors on the macro-phage derived foam cell formation.

In recent decades, the SR-A as A classic, is closely related to atherosclerosis of the receptor, has been attention by scholars. Scavenger receptors are thought to have adjustable macrophage's ability to intake modified LDL[9]. Macrophage derived foam cell formation is the most important pathological features of atherosclerosis, loaded it with lipid is very similar to the macrophages. SR-A is a kind of type ii membrane protein trimer and its corresponding ligands is very extensive, including apoptosis cells, glucan, after modification of LDL and gram-negative bacterial endotoxin, etc.[17,18]. Compared with the wild-type animals macrophages, knock out the SR-A macrophage of acetylated LDL intake reduced by 70%—80%[19]. In the process of atherosclerosis, inflammation factors can regulate the expression of SR-A and the formation of foam cells. However, a growing number of studies show that SR-A is not the only receptors involved in the process of atherosclerosis

It is well known that high levels of plasma LDL cholesterol and is closely related to higher incidence of atherosclerosis. LDL-R adjustable degradation in cholesterol in-take in the cells and plasma. Some studies have shown that LDL-R feedback regulation of cholesterol in the cell. The feedback system by regulating cholesterol synthesis and cholesterol intake to maintain the stability of cholesterol levels. The process by the LDL-R promoter sterol adjusting element 1 (sterol-regulatory element 1, SRE-1) and sterol adjusting element binding protein (sterol-regulatory element binding proteins, SREBPs) family of interaction and SREBP SREBP-1-2 control[20,21]. Studies have shown that inflammation factors such as element M IL-6, tumor and TNF α can raise LDL-R gene level expression. And, in the vascular smooth muscle cells and glomerular mesangial cells, inflammatory factors can disrupt the LDL-R feedback regulation, cellular cholesterol intake imbalance, resulting in the formation of foam cells. However, the role of LDL-R to the accumulation of cholesterol in has not been elucidated in Macrophages.

In this study, we study the effect of original LDL, inflammation, under the condition of the formation of macrophage foam cells derived and SR-A and the role of LDL-R. We demonstrate that the LPS induced cause inflammatory stress and TNF- α in time in accordance with the secretion of patience. LPS can increase the accumulation of cholesterol in RAW264.7 cells (*Figure 1*) and cause the formation of foam cells (*Figure 2*). To explore its molecular mechanism, we further study the LDL-R and SR RAW264.7 cells-A of the expression of mRNA and protein level. Under physiological conditions, LDL-R is sensitive to LDL lower. In RAW264.7 cells, the total cholesterol and cholesterol ester content in cells remain at a relatively constant level. However, inflammation may disrupt this feedback regulation, and by increasing the SR-A and the expression of LDL-R leads to formation of foam cells (*Figure 3–5*). Our results show that inflammation caused by LDL-R and SR-A raised on macrophage derived foam cell formation with synergy.

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