

Inhibitory Effect of Serum from Atherosclerotic Patients on Oxidized LDL Uptake by Macrophages

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ABSTRACT

Introduction: Atherosclerotic antigens can elicit an adaptive immune response, resulting in increased levels of autoantibodies in patient serum. However, the direct effects of patient serum and its autoantibodies on lipid uptake and foam cell formation remain unexplored. **Methods:** In this study, we investigated the effects of patient serum and its autoantibodies on macrophage lipid phagocytosis and foam cell formation, as well as assessed the antibody status against the whole antigen of foam cells in the serum. Human serum, which included samples from forty-five patients and eight healthy individuals, was co-cultured with the oxidized LDL (ox-LDL) and macrophages, and then the effect of the serum on inhibiting ox-LDL lipid engulfment by macrophages was assessed. Next, the binding ability of human serum antibodies to foam cells was evaluated using ELISA and immunofluorescence staining. **Results:** Our results indicated that 17.8% of patient serum samples demonstrate a significant inhibition of ox-LDL uptake by macrophages ($p < 0.05$). Among these samples, ELISA and immunofluorescence staining results further revealed that the circulating IgG and IgM levels against foam cell antigens were significantly higher in patients than in healthy individuals. The IC₅₀ value of purified antibodies from patients reached as low as 0.024 mg/mL in inhibiting ox-LDL uptake by macrophages, which is significantly lower than the IC₅₀ value of 0.054 mg/mL observed in healthy individuals. **Conclusion:** This study provides new insights into the protective role of patient serum and its autoantibody against atherosclerosis.

KEYWORDS

Atherosclerotic antigens; Autoantibody; Macrophage; Oxidized LDL; Foam cell

1. INTRODUCTION

Atherosclerosis, the underlying pathology behind major cardiovascular conditions like coronary artery disease, heart attacks, strokes, and peripheral artery disease, is a primary driver of global disability and mortality [1]. According to statistics, nearly 20.5 million people died from cardiovascular diseases in 2021 [2]. The development of atherosclerotic plaques is essentially a chronic inflammatory disease resulting from lipid metabolism imbalances and the deposition of lipid-rich foam cells in the arterial intima [3]. During the development of atherosclerosis, adaptive immune responses are triggered by autoantigens, resulting in the production of autoantibodies, such as those against ox-LDL and apoB-100 [4]. The production level of these autoantibodies has been suggested

as an indicator of atherosclerotic treatment, with serum anti-ApoB antibody levels serving as a marker for statin therapy in atherosclerotic conditions [5]. Autoantibodies that bind to epitopes may attenuate atherosclerotic plaque formation but can also accelerate the development of atherosclerosis, depending on the characteristics of the autoantigen and the subtype of antibody produced. In previous clinical studies, the presence of autoantibodies against apoB-100 and ox-LDL, including both IgG and IgM, revealed that serum IgM levels were negatively correlated with atherosclerosis severity, whereas IgG levels were positively correlated [6, 7]. However, animal studies indicate that the application of IgG antibodies against apoB-100 or ox-LDL can significantly inhibit atherosclerosis and demonstrate a protective effect [8-10]. The reasons for the inconsistencies between animal and clinical study results remain unknown.

Macrophages excessively engulf lipids such as ox-LDL and cannot metabolize or excrete them efficiently, leading to the formation of foam cells, which are key factors in the initiation and development of atherosclerotic plaques [5, 11]. Therefore, it is essential to investigate patient serum by focusing directly on lipid phagocytosis and foam cell formation, as these factors serve as a reference for evaluating the patient's ability to combat atherosclerosis. In addition, foam cells exhibit molecular characteristics that differ significantly from those of normal macrophages, making it theoretically possible to trigger an immune response and produce corresponding autoantibodies due to their immunogenicity. However, there have been no reports of autoantibodies specifically binding to foam cells. This study is designed to detect the potential capability of serum against lipid phagocytosis and foam cell formation, and also identify the autoantibodies against foam cells.

2. MATERIALS AND METHODS

2.1. Materials

Human low-density lipoprotein (ox-LDL) was brought from Guangzhou Yiyuan Biotech. Co., Ltd, while the Dio-labeled oxidized low-density lipoprotein (Dio-ox-LDL) was brought from G-clone (Beijing) Biotechnology Co., Ltd. HRP-conjugated Goat Anti-Human IgG (H+L), HRP-conjugated Goat Anti-Human IgM (H+L), and FITC-conjugated Goat Anti-Human IgG were purchased from Proteintech Group, Inc. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from AbBox Company, while the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was sourced from Beijing Solaibao Company. Oil Red O staining solution was purchased from Invitrogen. Protein L Plus Agarose Beads for antibody purification were obtained from Biolinked in Biotechnology Co., Ltd.

2.2. Serum Samples

All experimental data were anonymized to protect patient confidentiality. Serum samples were obtained from patients who visited the hospital between January 2023 and August 2024, including forty-five patients (aged 55 to 92 years, with an average age of 75 years) and eight healthy individual donors (aged 55 to 86 years, with an average age of 77 years). These serum samples were stored at -80 °C. Each patient was informed about the research purpose regarding the serum samples and their personal diagnostic information, and they signed an informed consent form.

2.3. Animal

Six-week-old BALB/c male mice (n = 10) were obtained from Chengdu Dashuo Laboratory Animal Co., Ltd. (Chengdu, China). Mice were housed under controlled environmental conditions (22 ± 3 °C, 56 ± 10% relative humidity) with *ad libitum* access to food and water. Health status was monitored daily throughout the study. Following a one-week acclimatization period, experimental procedures commenced. All animal protocols were approved by the Institutional Animal Care and Use

Committee (IACUC) of Sichuan University (Approval No. 20230307036). Anesthesia and euthanasia were induced via intraperitoneal injection of pentobarbital sodium, in compliance with European Directive 2010/63/EU.

2.4. Phagocytosis of ox-LDL and Foam Cells Preparation

The peritoneal macrophages were isolated from the peritoneal cavity of BALB/c mice and seeded in a 96-well plate at a density of 1.0×10^5 cells per well in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Australia). After 30 minutes of adhesion, the cells were washed with PBS to discard non-adhered cells and then cultured for 24 hours. Before adding lipids, LPS was introduced to stimulate the cells for 2 hours at a final concentration of 200 ng/mL. Subsequently, the ox-LDL concentrations were adjusted to 20 µg/mL, 50 µg/mL, and 100 µg/mL in RPMI 1640 medium. The modified solutions were then added to a 96-well plate and incubated overnight or co-incubated for 24 hours. According to the manufacturer's instructions, oil red staining was applied for 30 minutes, after which the wells were washed with PBS and observed for foam cells under a light microscope to determine the optimal lipid concentration for ox-LDL phagocytosis. For the preparation of foam cells, peritoneal macrophages were isolated and treated with LPS as described above. Then, ox-LDL was added to a dish (1×10^7 cells) with the optimal lipid concentration for 24 hours of phagocytosis.

2.5. Serum Effects on ox-LDL Uptake

The preparation of peritoneal macrophages and their treatment with LPS were performed as described in section 2.4. Next, the cells were incubated with or without serum (at a 1:20 dilution) in the presence of ox-LDL (50 µg/mL) for 24 hours. Finally, the cells were stained with Oil Red O for the assessment of ox-LDL phagocytosis.

2.6. ELISA Analysis

Foam cells were collected and sonicated, and the protein concentration of the cell lysate was determined using the Bradford method. To prepare the antigen-coated ELISA plate, 100 µL of cell lysate (10 µg/mL) or 2 µg of ox-LDL were added to the ELISA plate and incubated overnight at 4 °C. Next, washed the ELISA plates with PBST and then blocked with 3% BSA for 2 hours at room temperature (RT), followed by incubation with serum at a 1:50 dilution for 2 hours. The plates were washed again and incubated with HRP-labelled anti-human IgG (at a 1:10000 dilution) or HRP-labelled anti-human IgM (at a 1:5000 dilution) for 1 hour at RT. The substrate TMB (50 µL) was then added to the plates and incubated for 10 minutes at RT after washing three times with PBST. Finally, 25 µL of 2M H₂SO₄ was added to each well, and the optical density (OD) was immediately read at 450 nm.

2.7. Immunofluorescence Staining

The foam cells were prepared as described in section 2.4. Immunofluorescence staining was then conducted as follows. Briefly, foam cells were fixed in 4% paraformaldehyde for 10 minutes and washed with PBS. The cells were blocked with 3% BSA for 2 hours at RT, followed by incubation with serum at a 1:50 dilution for 2 hours. After washing with PBS, FITC-conjugated goat anti-human IgG (1:250 dilution) was added, and the cells were incubated at RT for 45 minutes in the dark. Subsequently, the cells were washed with PBS, and 200 µL of DAPI working solution (0.5 µg/mL) was added and stained for 5 minutes at RT. The staining solution was discarded, the cells were washed with PBS, and then observed and photographed under fluorescence microscopy. The relative quantitative analysis of fluorescence intensity was performed using Image Pro Plus software.

2.8. Antibody Purification From Serum

Total antibodies from serum, including IgM and IgG, were purified using Protein L Plus Agarose Beads according to the manufacturer's protocol. All procedures were conducted at 4°C. Serum samples included eight patient samples (P4, P6, P8, P26, P33, P34, P48, P49) and eight healthy donor serum samples (HD1–HD8). After purification, these purified antibodies were verified using SDS-PAGE.

2.9. Effects of Antibody on the Uptake of ox-LDL

Macrophages were then incubated with fluorescently labeled DiO-ox-LDL (20 µg/mL) for 4 hours at 37 °C in the presence of purified antibody at concentrations ranging from 0.0 to 1.0 mg/mL. The internalized ox-LDL in the cells was fixed with 4% paraformaldehyde for 10 minutes, washed with phosphate-buffered saline (PBS), and then stained with 100 µL of DAPI working solution (1µg/mL) for 3 minutes at room temperature (RT) to label nuclei, followed by further PBS washes. Internalized Dio-ox-LDL was visualized using fluorescence microscopy, with six random fields captured per experimental condition. Fluorescence intensity was quantified using Image Pro Plus software, and antibody IC₅₀ values were derived from dose-response curves.

2.10. Statistical Analysis

Experimental data are presented as means ± standard deviations (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare differences among more than two groups with SPSS 22.0 software. For comparisons between two groups, the unpaired t-test or nonparametric Mann-Whitney test was used. p-values < 0.05 and < 0.01 indicate significant and very significant differences, respectively. Each experiment was conducted three times.

3. RESULTS

3.1. Optimal Lipid Concentration for ox-LDL Phagocytosis

The presence of FBS significantly increased the background signal of oil red staining compared to serum-free medium, prompting lipid phagocytosis experiments to be performed in low serum culture (containing 2% FBS). Co-incubation with macrophages at varying concentrations of ox-LDL showed that 50 µg/mL was sufficient for effective lipid phagocytosis (Figures 1a and 1b). Under the light microscope, macrophages exhibited excessive phagocytosis of ox-LDL lipids, resulting in numerous lipid droplets inside the cells, an enlarged cytoplasm, and even cell death, all of which demonstrate the typical characteristics of foam cells.

3.2. Inhibition Effects of Serum on ox-LDL Phagocytosis

Generally, the oil red staining showed that the patient serum treatment group demonstrated lower ox-LDL uptake than the normal serum treatment group, compared to the positive control (Figure 2b). Among these patients, 17.8% of serum samples (8/45) significantly inhibited macrophage phagocytosis of ox-LDL and foam cell formation ($p < 0.05$, Figure 2a). Notably, the serum samples from patients P4, P6, and P8 exhibited a strong inhibition of macrophage phagocytosis of ox-LDL, with reductions exceeding 50% ($p < 0.01$, Figures 2c and 2d).

3.3. Serum Autoantibodies Detection

ELISA results showed that the levels of IgG and IgM against foam cells in the serum of patients were significantly increased compared to those in normal human serum ($p < 0.05$, Figure 3a). Additionally,

we also detected IgG and IgM against ox-LDL, which showed a similar elevation to the antibodies against foam cells ($p < 0.05$, Figure 3b). To further confirm these findings, immunofluorescence staining was performed on foam cells using serum from patients and healthy serum, respectively. As shown in Figure 3c and 3d, immunofluorescence studies confirmed significant binding of serum IgG antibodies to foam cells (Figures 3c and 3d).

3.4. Inhibitory Effects of Purified Antibodies on Lipid Uptake

As shown in Figures 4a and 4b, SDS-PAGE analysis confirmed successful purification of immunoglobulins from the pooled serum of eight patients and eight healthy donors, as evidenced by clearly visible heavy- and light-chain bands. Antibodies from patient serum inhibited DiO-labeled ox-LDL uptake by macrophages in a dose-dependent manner (Figure 4c). Notably, antibody preparations from patient serum produced a significantly greater inhibition of ox-LDL uptake than those from healthy donors ($p < 0.05$) (Figure 4d). Quantitative fluorescence analysis revealed that the half-maximal inhibitory concentration (IC₅₀) of purified antibodies from patients was 24 $\mu\text{g/mL}$, compared with 54 $\mu\text{g/mL}$ for antibodies from healthy donors (Figure 4d).

4. DISCUSSION

In this study, we firstly evaluated the inhibitory effects of patient serum on lipid phagocytosis by macrophages and foam cell formation. We found that 17.8% of patient serum possesses the significant capability of inhibiting macrophage phagocytosis of ox-LDL, suggesting a protective role of patient serum in atherosclerosis. The serum

contains many components. Furthermore, when foam cells or ox-LDL were used as coating antigens, patient serum showed increased levels of both IgG and IgM autoantibodies. These findings highlight the involvement of specific immune responses in atherosclerosis, where antigens and their cognate autoantibodies can trigger autoimmune reactions with an atheroprotective role [12].

Previous studies have suggested that there are mainly two subtypes of antibodies: IgM and IgG, which target atherosclerotic autoantigens such as ox-LDL, apoB-100, and apolipoprotein A-I. The IgM level is generally considered to have an atheroprotective role, while the IgG level is associated with a pro-atherogenic role [6, 7, 13]. B-1 and B-2 cells are developmentally and functionally distinct subsets of B lymphocytes. B-1 cells are responsible for the production of the main circulating IgM in a T-cell-independent manner [13, 14]. However, there is uncertainty about the role of B-2 cells and IgG levels in atherosclerosis [15]. Much evidence suggests a pro-atherogenic role for B-2 cells, but some experimental data also support a protective role [16-18]. Here, we evaluated the recognition and binding of serum antibodies to normal macrophage and foam cell antigens using ELISA and immunofluorescence staining. Patient serum exhibited significantly stronger IgG/IgM binding signals to foam cells (FCs) compared to serum from healthy donors, which showed only weak binding. This demonstrates the presence of circulating IgG and IgM antibodies targeting autoantigens derived from foam cells in patients. Interestingly, we additionally detected IgG antibodies specifically binding to ox-LDL in patient serum. Nevertheless, the pathophysiological role and mechanistic links between these FC-specific IgG antibodies and atherosclerosis remain to be fully elucidated.

Foam cells differ significantly from macrophages in terms of cell phenotype and molecular characteristics [19, 20], which may allow them to activate adaptive immunity as autoantigens. In previous animal studies, foam cells served as whole antigens to immunize mice, leading to the production of foam cell-specific antibodies that inhibit plaque formation [21]. As a result, apoE^{-/-} mice gained immune protection against atherosclerosis. It should be noted that the epitopes of foam cell autoantigens are complex and differ significantly from those of autoantigens like apoB-100 and ox-LDL, resulting in various types of autoantibodies. There are several limitations in this study. When reviewing the patient visit data, it is challenging to compare and analyze the lipid phagocytosis data

with the patient's condition due to the lack of quantitative data, the absence of a drug use history, and variations in age, gender, and lifestyle habits. However, given the important role of foam cells in the formation of atherosclerotic plaques, it would be worthwhile to use single B cell sorting in future studies to identify autoantibodies and evaluate its capability against atherosclerosis.

5. CONCLUSIONS

A subset of patient serum significantly inhibited ox-LDL uptake by macrophages and reduced foam cell formation ($p < 0.05$). This effect is at least partially attributable to elevated circulating IgG and IgM autoantibody levels against atherosclerotic antigens in these patient. This study provides new insights into the protective role of patient serum and its autoantibody against atherosclerosis, and it would be worthwhile to use single B-cell sorting to further identify these autoantibodies and evaluate their anti-atherosclerotic potential.

AUTHORS' CONTRIBUTIONS

Zhi Zhang: Conceptualization, Funding acquisition, Supervision, Validation, Project administration, Resources, Writing - original draft, Writing - review & editing. Shiyuan Deng: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft. Weihang He and Yuzhen Liao: Formal analysis, Investigation, Resources. Meifang Du: Methodology. Rania G. Elbaiomy and Xiaohong Jian: Resources, Supervision, Validation.: Resources, Supervision, Validation. Yongmei Xie: Formal analysis, Writing - review & editing. Yi Wang: Data curation, Formal analysis, Methodology, Investigation, Writing - review & editing. Zaixin Li: Resources, Supervision, Validation, Writing - review & editing. Data availability

The data supporting this study cannot be publicly shared due to ethical restrictions (Ethics Approval No. 2023KY004KS). Access requests may be directed to the corresponding author, subject to institutional ethics review and compliance with applicable regulations.

ACKNOWLEDGEMENTS

This work was supported by the Sichuan Province Science and Technology Support Program (Grant No. 2023NSFSC0586, 24GJHZ0088).

DECLARATIONS OF ETHICS APPROVAL

This study involving human serum samples was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Fushun County People's Hospital (Approval No. 2023KY004KS). Written informed consent was obtained from all participants prior to sample collection. All experimental data were anonymized to protect patient confidentiality.

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