OxLDL/β2GPI/anti-β2GPI Ab complex induces inflammatory activation via the TLR4/NF-κB pathway in HUVECs

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Abstract. Patients with antiphospholipid syndrome have been identified to have higher incidence rates of atherosclerosis (AS) due to the elevated levels of anti-β2-glycoprotein I (β2GPI) antibody (Ab). Our previous studies revealed that the anti-β2GPI Ab formed a stable oxidized low-density lipoprotein (oxLDL)/β2GPI/anti-β2GPI Ab complex, which accelerated AS development by promoting the accumulation of lipids in macrophages and vascular smooth muscle cell. However, the effects of the complex on endothelial cells, which drive the initiation and development of AS, remain unknown. Thus, the present study aimed to determine the proinflammatory roles of the oxLDL/β2GPI/anti-β2GPI Ab complex in human umbilical vein endothelial cells (HUVECs) in an attempt to determine the underlying mechanism. Reverse transcription-quantitative PCR, enzymy-linked immunosorbent assay, western blotting and immunofluorescence staining were performed to detect the expressions of inflammation related factors and adhesion molecules. Monocyte-binding assay was used to investigate the effects of oxLDL/β2GPI/anti-β2GPI Ab complex on monocyte adhesion to endothelial cells. The results demonstrated that the oxLDL/β2GPI/anti-β2GPI Ab complex upregulated the expression of Toll-like receptor (TLR)4 and the levels of NF-κB phosphorylation in HUVECs, and subsequently enhanced the expression levels of inflammatory cytokines, including TNF-α, IL-1β and IL-6, as well as those of adhesion molecules, such as intercellular adhesion molecule 1 and vascular adhesion molecule 1. In addition, the complex facilitated the recruitment of monocytes by promoting the secretion of monocyte chemotactic protein 1 in HUVECs. Notably, the described effects of the oxLDL/β2GPI/anti-β2GPI Ab complex in HUVECs were abolished by either TLR4 or NF-κB blockade. In conclusion, these findings suggested that the oxLDL/β2GPI/anti-β2GPI Ab complex may induce a hyper-inflammatory state in endothelial cells by promoting the secretion of proinflammatory cytokines and monocyte recruitment, which was discovered to be largely dependent on the TLR4/NK-κB signaling pathway.

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Abbreviations: APS, antiphospholipid syndrome; AS, atherosclerosis; oxLDL, oxidized low-density lipoprotein; β2GPI, β2 glycoprotein I; anti-β2GPI Ab, anti-β2 glycoprotein I antibody; VSMCs, vascular smooth muscle cells; TLR4, Toll-like receptor 4; HUVECs, human umbilical vein cells; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1; MCP-1, monocyte chemoattractant protein 1; LPS, lipopolysaccharide

Key words: oxLDL/β2GPI/anti-β2GPI Ab, HUVECs, inflammatory cytokines, adhesion molecules, TLR4, NF-κB

Introduction

Atherosclerosis (AS), which is characterized by hyperlipidemia, lipid plaque formation and an accompanying complex vascular inflammatory response, is considered as the major contributor for the development of cardiovascular disease worldwide (1-3). Accumulating evidence has revealed the role of autoimmunity in AS (4). Clinical studies have identified that patients with autoimmune diseases, such as antiphospholipid syndrome (APS) or systemic lupus erythematous, experience significant morbidity and mortality due to AS (5,6).

Endothelial inflammation was discovered to significantly contribute to the initiation and progression of AS by damaging the vascular wall, promoting monocyte adhesion and infiltration, and accelerating lipid accumulation in the subendothelial space (7,8). IL-1β, IL-6 and TNF-α, which are secreted by endothelial cells, are reported to be closely associated with arterial damage and vascular inflammation during the early stages of AS (9). In addition, adhesion molecules released from endothelial cells, such as intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule

(VCAM)-1, are observed to serve important roles in the recruitment of circulating monocytes (10).

Oxidized low-density lipoprotein (oxLDL) is known to be an important lipid component in AS, and exerts roles throughout almost every stage of the disease (11). OxLDL accelerates AS progression by triggering vascular inflammation and lipid accumulation, which are the important pathological factors involved in the initiation and development of AS (12). However, previous clinical evidence has indicated that the oxLDL/β2-glycoprotein I (β2GPI) complex may be a more substantial indicator of cardiovascular complications compared with oxLDL alone in patients with APS (13,14). Previous studies have also reported that the increased risk of AS in patients with APS was mainly due to β2GPI and its autoantibodies (4-6). For example, our previous *in vivo* study confirmed that the presence of the anti-β2GPI antibody (Ab) accelerated plaque formation in ApoE-/- mice (15). In addition, an *in vitro* study found that the oxLDL/β2GPI/anti-β2GPI Ab complex induced the proatherogenic activation of vascular smooth muscle cells (VSMCs) by enhancing their migratory abilities and the secretion of active molecules (16).

Similar to the majority of the members of the pattern recognition receptor family, Toll-like receptor (TLR) 4 is capable of recognizing a wide range of danger-associated molecules, including microbial components, such as lipopolysaccharide (LPS) and modified endogenous molecules, including oxLDL (17). Upon recognition, the invaded pathogens are eliminated by triggering the inflammatory response (18). Several studies have revealed that TLR4 is involved in antiphospholipid Ab-mediated thrombosis and the activation of endothelial cells, which could activate p38 MAPK and NF-κB, leading to the subsequent upregulation of various genes and the production of proinflammatory cytokines in patients with APS (19-21). Moreover, the activation of the TLR4/NF-κB signaling pathway was discovered to promote the secretion of inflammatory cytokines and the lipid accumulation of macrophages during the pathogenesis of AS (22,23).

Our previous studies have demonstrated that the oxLDL/β2GPI/anti-β2GPI Ab complex induced the differentiation of macrophages to foam cells and altered the proliferation and apoptosis of VSMCs via a biphasic effect (24,25). Considering the crucial role of inflammatory activation of endothelial cells in AS development, the effect of the oxLDL/β2GPI/anti-β2GPI Ab complex on endothelial cells requires further investigation. Therefore, the present study aimed to evaluate the expression levels of inflammatory cytokines, adhesive molecules and chemokines in human umbilical vein endothelial cells (HUVECs) in the presence of the oxLDL/β2GPI/anti-β2GPI Ab complex, in addition to determining the ability of HUVECs to recruit monocytes and identifying the role of the TLR4/NF-κB signaling pathway in these processes.

Materials and methods

Cell culture. HUVECs were obtained from the Shanghai Institutes for Biological Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS (Biological Industries), 4.5 g/l glucose, 1% glutamine and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained at 37˚C and 5% CO_2 in a humidified incubator until 90% confluence.

THP-1, a human monocytes cell-line, was purchased from Shanghai Institutes for Biological Sciences. THP-1 monocytes were cultured at 37˚C in a 5% CO_2/95% humidified air incubator (Thermo Fisher Scientific, Inc.) in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Biological Industries) and 1% penicillin-streptomycin antibiotics (Gibco; Thermo Fisher Scientific, Inc.).

Preparation and identification of stimuli. Following 12 h of serum deprivation, HUVECs were stimulated with DMEM, oxLDL (50 μg/ml; cat. no. YB-002; http://www.yiyuanbiotech.com/PRO.asp?id=562#section2; Guangzhou Yiyuan Biotech. Co. Ltd.), oxLDL(50 μg/ml)/β2GPI (100 μg/ml; cat. no. G9173; Sigma-Aldrich; Merck KGaA) complex, oxLDL (50 μg/ml)/anti-β2GPI Ab (100 μg/ml; cat. no. HPA001654; Sigma-Aldrich; Merck KGaA) complex, oxLDL (50 μg/ml)/β2GPI (100 μg/ml)/anti-β2GPI Ab (100 μg/ml) complex (24-26) or LPS (500 ng/ml; Sigma-Aldrich; Merck KGaA) at 37˚C for 2 or 24 h.

For preparation of the complex of oxLDL/β2GPI, 50 μg oxLDL and 100 μg β2GPI were added to 1 ml DMEM (with 10% FBS and 1% antibiotics), and then incubated at 37˚C and pH 7.4 for 16 h, as previously described (24,25). The complex of oxLDL/anti-β2GPI Ab and oxLDL/β2GPI/anti-β2GPI Ab were prepared by incubating oxLDL (50 μg/ml) or oxLDL (50 μg/ml)/β2GPI (100 μg/ml) complex with anti-β2GPI Ab (100 μg/ml) at 37˚C for 30 min (26). The concentrations and incubation times of the aforementioned reagents were selected according to established protocols and previous studies (24-27).

After performing the aforementioned incubations, the binding of complex was confirmed via ELISA according to previous studies (26,27). Firstly, anti-human apoB-100 Ab (cat. no. SAB2500080; Sigma-Aldrich; Merck KGaA) was adsorbed onto microtiter plates by incubating at 8 mg/ml (dissolved in Carbonate buffered saline, 50 μl/well) at 4˚C overnight. After blocking with PBS containing 10% Newborn Calf Serum (NBCS; cat. no. 80230-6412; Zhejiang Tianhang Biotechnology Co., Ltd.) at 37˚C for 1 h, the samples (oxLDL/β2GPI, oxLDL/anti-β2GPI Ab or oxLDL/β2GPI/anti-β2GPI Ab) diluted (1:100) with PBS containing 10% NBCS were added to the wells (100 μl/well) to be incubated at 37˚C for 2 h. The blank control well was added with PBS containing 10% NBCS. The wells were then incubated with anti-β2GPI Ab at 37˚C for 30 min and then incubated with horseradish peroxidase (HRP)-labeled anti-IgG Ab [cat. no. 05-4030-05; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] at room temperature (RT) for 2 h (for Blank control and oxLDL/β2GPI) or HRP-labeled anti-IgG Ab (for oxLDL/anti-β2GPI Ab and oxLDL/β2GPI/anti-β2GPI Ab) at RT for 2 h. Extensive washing between steps was performed with PBS containing 0.05% Tween-20. Color was developed with tetramethylbenzidine (TMB) and H_2O_2 at RT for 15 min. The reaction was terminated, and optical density at 450 nm was measured.

For the inhibition of TLR4 and NF-κB, the cells were pretreated with 5 μM TAK-242 (Invitrogen; Thermo Fisher Scientific, Inc.) or 20 μM 2-phosphonobutane-1,2,4,

-tricarboxylic acid (PDTC; Sigma-Aldrich; Merck KGaA) at 37˚C for 6 or 1.5 h, respectively, according to the manufacturer's protocols.

Reverse transcription-quantitative PCR (RT-qPCR). The cells were seeded at a density of $2x10^6$ cells/well into a 6-well plate and starved in serum-free media for 12 h prior to stimulation. Total RNA was extracted from HUVECs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the HiScript™ First-strand cDNA Synthesis kit (Vazyme Biotech Co., Ltd.) at 50˚C for 15 min and followed by 85˚C for 2 min. oligo dT-primers (Sangon Biotech, Co., Ltd.) were used for the reverse transcription alongside 1 μg total RNA in a 10 μl reaction volume. The expression levels of target mRNAs in the HUVECs were subsequently analyzed via qPCR using SYBR-Green I dye (Vazyme Biotech Co., Ltd.). The primer pairs used for the qPCR are listed in Table SI. The following thermocycling conditions were used for the amplification run: Initial denaturation at 95˚C for 30 sec; followed by 39 cycles at 56˚C (VCAM-1)/58˚C (ICAM-1, IL-1β and IL-6)/60˚C [TNF-α, monocyte chemotactic protein (MCP-1), TLR4 and β-actin] for 30 sec and extension at 72˚C for 30 sec. The expression levels of the target genes were quantified using the $2^{-\Delta\Delta Cq}$ method (28) and normalized to the control gene, β-actin.

Western blotting. The cells were stimulated with different stimuli as aforementioned and then total protein was extracted using RIPA lysis buffer (cat. no. P0013K; Beyotime Institute of Biotechnology) for 1 h in an ice bath. Cellular protein concentrations were determined using a BCA Protein assay kit (cat. no. P0011; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Protein samples (100 μg) were separated by electrophoresis on 8-10% polyacrylamide gels and the separated proteins were subsequently transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked in fresh 5% dry non-fat milk diluted in TBS-0.05% Tween-20 (TBS/T) for 1 h at room temperature. After being washed with TBS/T for 15 min three times, the membranes were then incubated with the following primary Abs at 4˚C overnight: Monoclonal rabbit anti-TLR4 (1:500; cat. no. sc-76B357.1; Santa Cruz Biotechnology, Inc.), monoclonal rabbit anti-phosphorylated (p)-NF-κB-p65 Ser536 (1:1,000; cat. no. 3033; Cell Signaling Technology, Inc.), monoclonal rabbit anti-NF-κB p65 (1:1,000; cat. no. 8242; Cell Signaling Technology, Inc.), monoclonal rabbit anti-p-AKT (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), monoclonal rabbit anti-AKT (1:1,000; cat. no. 4685; Cell Signaling Technology, Inc.) and polyclonal anti-β-actin (1:5,000; cat. no. AP0060; Bioworld Technology). Following the primary Ab incubation, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary Ab (1:5,000; cat. no. BS13278; Bioworld Technology) at RT for 1 h after washing three times with TBS/T for 15 min. Protein bands were visualized using enhanced ECL western blotting detection reagent (Cytiva) on an Image Quant LAS 4000 imager, and the densitometric analysis was performed using LANE 1D (version 4.0; Beijing Sage Creation Science Co., Ltd.).

ELISAs. The cells were seeded at a density of $1.0x10^5$ cells/well into 24-well plates and treated with different stimuli as previously described following serum starvation for 12 h. For TLR4 or NF-κB inhibition, cells in specific wells were pretreated with TAK-242 (5 μM) for 6 h or PDTC (20 μM) at 37˚C for 1.5 h prior to the other treatments. Following stimulation, the cell culture supernatants were centrifuged for 10 min at 300 x g at 4˚C. The concentrations of TNF-α, IL-1β, IL-6 and MCP-1 in the cell culture supernatants were analyzed using TNF-α [cat. no. 70-EK182-96; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.], IL-1β [cat. no. 70-EK101B-96; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.], IL-6 [cat. no. 70-EK106/2-96; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] and MCP-1 [cat. no. 70-EK187-96; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] according to the manufacturer's protocols. The concentrations of the cytokines were expressed as pg/ml.

Immunofluorescence staining. HUVECs were seeded at a density of $1.0x10^5$ cells/well into 24-well plates. Following 24 h of incubation with the stimuli, the cells were fixed in 4% paraformaldehyde at 4˚C for 30 min and then washed gently with PBS three times. The cells were subsequently blocked with 5% BSA [cat. no. A600332; Sangon Biotech (Shanghai) Co., Ltd.] at RT for 1 h and then incubated overnight at 4˚C with the following primary Ab: Anti-ICAM-1 (1:500; cat. no. ab2213; Abcam) and anti-VCAM-1 (1:500; cat. no. ab134047; Abcam). Subsequently, the cells were incubated with an AF488-conjugated secondary Ab (1:500; cat. no. K0034G-AF488; Beijing Solarbio Science & Technology Co., Ltd.). The nuclei were stained with DAPI (cat. no. C0065, Beijing Solarbio, Science & Technology Co., Ltd.) for 15 min at 37˚C. The expression levels of the proteins were observed and imaged using the fluorescent microscopes on the BioTek Cytation 5 Cell Imaging Multi-Mode reader (BioTek Instruments, Inc.).

Monocyte adhesion assay. HUVECs were stimulated with different stimuli as aforementioned for 24 h and then rinsed with PBS. Subsequently, $1x10^5$ THP-1 cells/well, which were seeded into 24-well plates and pre-stained with 5 μM Dil (cat. no. D9780; Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 30 min, were added to the HUVEC cultures and incubated at 37˚C and 5% CO_2 for 30 min in a humidified incubator. After being washed with PBS, THP-1 cells that were adhered to HUVECs were visualized and imaged under a fluorescence microscope at a magnification of x100 (Leica Microsystems GmbH). The Dil (red fluorescence) intensity in each field of view was calculated using ImageJ 2x 2.1 software (National Institute of Health), which reflected the number of adhered THP-1 cells.

Statistical analysis. All the experiments were repeated ≥3 times for each experimental condition and the representative results are presented. Normally distributed data are expressed as the mean ± SEM. Differences between control and experimental conditions were assessed using the one-way ANOVA with Tukey's multiple group comparison test. The two-factor treatment results were analyzed using a two-way ANOVA with Bonferroni's test. All the statistical analyses were performed using SPSS 24.0 software (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

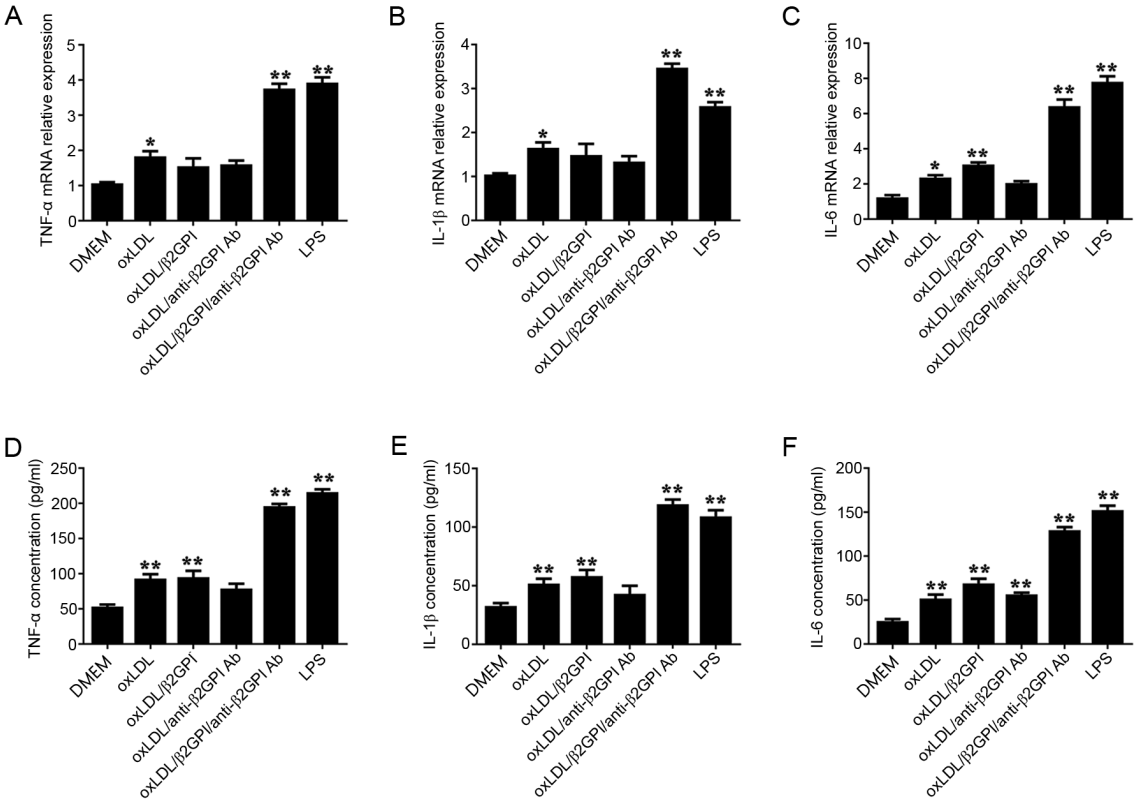

Figure 1. Effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression levels of the inflammatory cytokines TNF-α, IL-1β and IL-6. HUVECs were treated with DMEM, oxLDL, oxLDL/β2GPI complex, oxLDL/anti-β2GPI Ab complex, oxLDL/β2GPI/anti-β2GPI Ab complex or LPS for 2 h (for qPCR) or for 24 h (for ELISA). Total RNA was extracted from HUVECs and the mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 were analyzed using RT-qPCR. The cell culture supernatants of the HUVECs were collected for the detection of (D) TNF-α, (E) IL-1β and (F) IL-6 concentrations using ELISAs. *P<0.05, **P<0.01 vs. DMEM group. OxLDL, oxidized low-density lipoprotein; β2GPI, β2 glycoprotein I; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR; HUVECs, human umbilical vein endothelial cells; Ab, antibody.

Results

Effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression levels of inflammatory cytokines (TNF-α, IL-1β and IL-6) in HUVECs. To investigate the role of the oxLDL/β2GPI/anti-β2GPI Ab complex in endothelial inflammation, HUVECs were treated with DMEM, oxLDL, oxLDL/β2GPI, oxLDL/anti-β2GPI Ab, oxLDL/β2GPI/anti-β2GPI Ab complex or LPS for 2 or 24 h. The oxLDL/β2GPI, oxLDL/anti-β2GPI Ab and oxLDL/β2GPI/anti-β2GPI Ab complex were prepared and validated prior to experiment (P<0.01; Fig. S1). Following incubation with the stimuli, the mRNA and protein expression levels of TNF-α, IL-1β and IL-6 were analyzed using RT-qPCR and ELISAs, respectively. Treatment of cells with oxLDL and the oxLDL/β2GPI/anti-β2GPI Ab complex significantly upregulated the mRNA and protein expression levels of TNF-α (Fig. 1A and D), IL-1β (Fig. 1B and E) and IL-6 (Fig. 1C and F) compared with the DMEM control group (P<0.01; Fig. 1). The expression levels of the inflammatory cytokines in the oxLDL/β2GPI and oxLDL/anti-β2GPI Ab groups were also upregulated, although not all the changes were statistically significant. The effects of the oxLDL/β2GPI/anti-β2GPI Ab complex treatment were more notable compared with the oxLDL, oxLDL/β2GPI and oxLDL/anti-β2GPI Ab groups, although similar results were observed using LPS.

OxLDL/β2GPI/anti-β2GPI Ab complex upregulates the expression of TLR4 and the phosphorylation of NF-κB in HUVECs. TLR4 and NF-κB signaling pathways have been widely confirmed to serve critical roles in the pathological process of AS (22,29). In the present study, TLR4 expression levels and the phosphorylation of NF-κB were analyzed during the endothelial inflammation induced by the oxLDL/β2GPI/anti-β2GPI Ab complex. The oxLDL/β2GPI/anti-β2GPI Ab complex significantly upregulated the mRNA and protein expression levels of TLR4 in HUVECs compared with the DMEM group, and a similar effect was observed following LPS treatment (P<0.01; Fig. 2A and C). Moreover, the phosphorylation levels of NF-κB p65 at Ser536 were significantly increased following the incubation with the oxLDL/β2GPI/anti-β2GPI Ab complex or LPS compared with the DMEM group (P<0.01; Fig. 2D). As the other down-stream members of TLR4 signaling, AKT was also found to be phosphorylated in oxLDL/β2GPI/anti-β2GPI Ab complex or LPS-treated HUVECs compared with the DMEM group (P<0.05; Fig. S2).

Involvement of the TLR4/NF-κB signaling pathway in the oxLDL/β2GPI/anti-β2GPI Ab complex-induced expression of inflammatory cytokines in HUVECs. To further determine the involvement of TLR4 and NF-κB in the oxLDL/β2GPI/anti-β2GPI Ab complex-induced expression of inflammatory cytokines in HUVECs, TAK-242 or PDTC were used to inhibit their signal transduction,

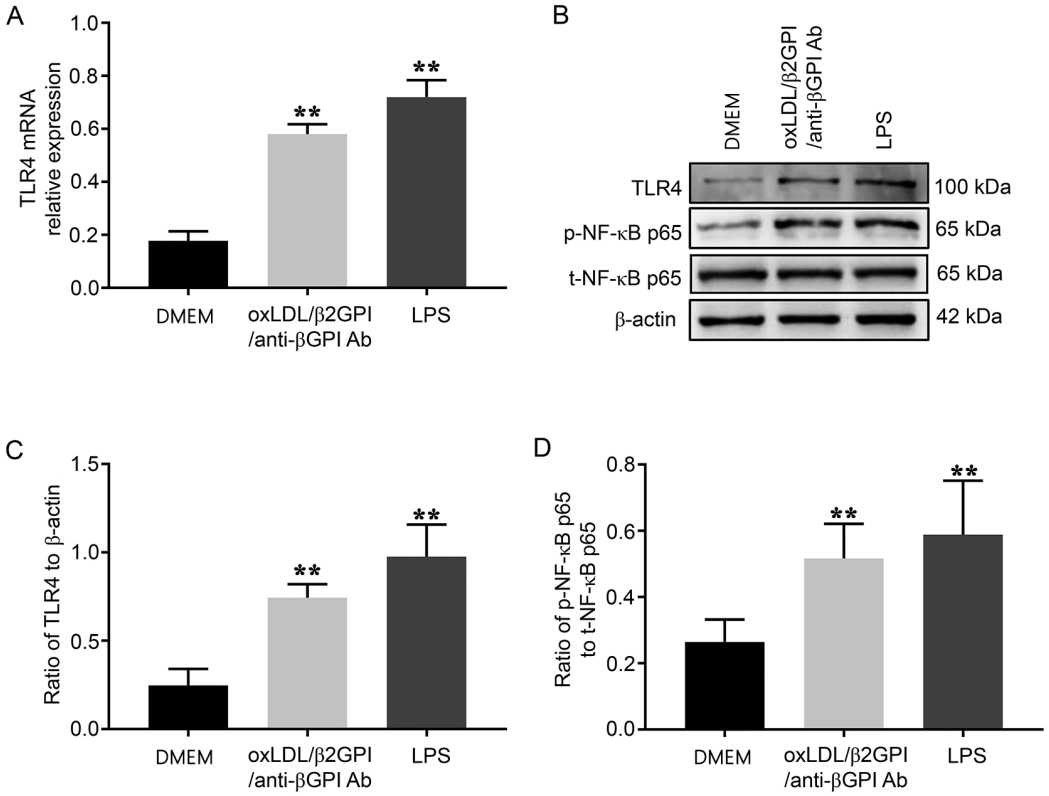

Figure 2. Effect of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression of TLR4 and the phosphorylation levels of NF-κB. Human umbilical vein endothelial cells were treated with DMEM, oxLDL/β2GPI/anti-β2GPI complex or LPS. (A) mRNA expression levels of TLR4 were analyzed using reverse transcription-quantitative PCR. (B) Western blotting was used to analyze the expression of TLR4 and the phosphorylation levels of NF-κB p65. (C) Densitometric semi-quantification of the protein expression of TLR4. (D) Densitometric semi-quantification of the phosphorylation levels of NF-κB p65. **P<0.01 vs. corresponding DMEM group. OxLDL, oxidized low-density lipoprotein; β2GPI, β2 glycoprotein I; Ab, antibody; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; p-, phosphorylated; t-, total.

respectively. The results demonstrated that the upregulated mRNA expression levels TNF-α, IL-1β and IL-6 induced by the oxLDL/β2GPI/anti-β2GPI Ab complex were blocked by pretreating the cells with TAK-242 or PDTC (P<0.01; Fig. 3A-C). Identical results were observed in the protein expression (P<0.01; Fig. 3D-F). The inhibition of NF-κB imposed weaker effects on abrogating the induction of IL-1β and IL-6, as well as the protein secretion of TNF-α compared with the inhibition of TLR4 (P<0.01; Fig. 3B, D, E and F; P<0.05; Fig. 3C). Therefore, these results indicated that the blockade of the TLR4/NF-κB signaling pathway may effectively inhibit the expression levels of TNF-α, IL-1β and IL-6 induced by the oxLDL/β2GPI/anti-β2GPI Ab complex.

OxLDL/β2GPI/anti-β2GPI Ab complex induces the expression of adhesion molecules and the involvement of the TLR4/NF-κB signaling pathway. Endothelial activation includes the expression of adhesion molecules (ICAM-1 and VCAM-1) on cell surface (8). Adhesion molecules are essential in endothelial inflammatory responses due to their ability to affect leukocyte adherence and to activate the vascular endothelium, which perpetuates a chronic proinflammatory state and fosters the progression of atherosclerotic lesion (10,30). Both oxLDL/β2GPI/anti-β2GPI Ab complex and LPS stimulation were able to significantly upregulate the mRNA expression levels of ICAM-1 and VCAM-1 compared with the DMEM group (P<0.01; Fig. 4A and B). Similarly, the protein expression levels of ICAM-1 and VCAM-1 in presence of the oxLDL/β2GPI/anti-β2GPI Ab complex and LPS were notably upregulated compared with that in DMEM group (Fig. 4C and D).

Subsequently, cells were pretreated with TAK-242 or PDTC prior to the other treatments. The results demonstrated that TAK-242 and PDTC pretreatment blocked the upregulation in the expression levels of ICAM-1 and VCAM-1 induced by the oxLDL/β2GPI/anti-β2GPI Ab complex or LPS compared with the groups without inhibitors (P<0.01; Fig. 4). The effects produced by TAK-242 on the induction of ICAM-1 were found to be more significant compared with those induced by PDTC (P<0.05; Fig. 4A), and there was no difference between these two inhibitors pretreatment on the induction of VCAM-1(P>0.05; Fig. 4B).

Involvement of the TLR4/NF-κB signaling pathway in the oxLDL/β2GPI/anti-β2GPI Ab complex-induced upregulation of MCP-1 expression and the adhesion of THP-1 to HUVECs. MCP-1 has been discovered to trigger monocytes, which circulate in the blood to aggravate vascular inflammation and induce thrombosis in the plaque (31). To investigate whether the induction of the oxLDL/β2GPI/anti-β2GPI Ab complex could increase monocyte adhesion, the adhesion of Dil-stained THP-1 cells to HUVECs was analyzed using an improved monocyte-EC adhesion assay, in addition to evaluating the expression of MCP-1. The results indicated that the oxLDL/β2GPI/anti-β2GPI

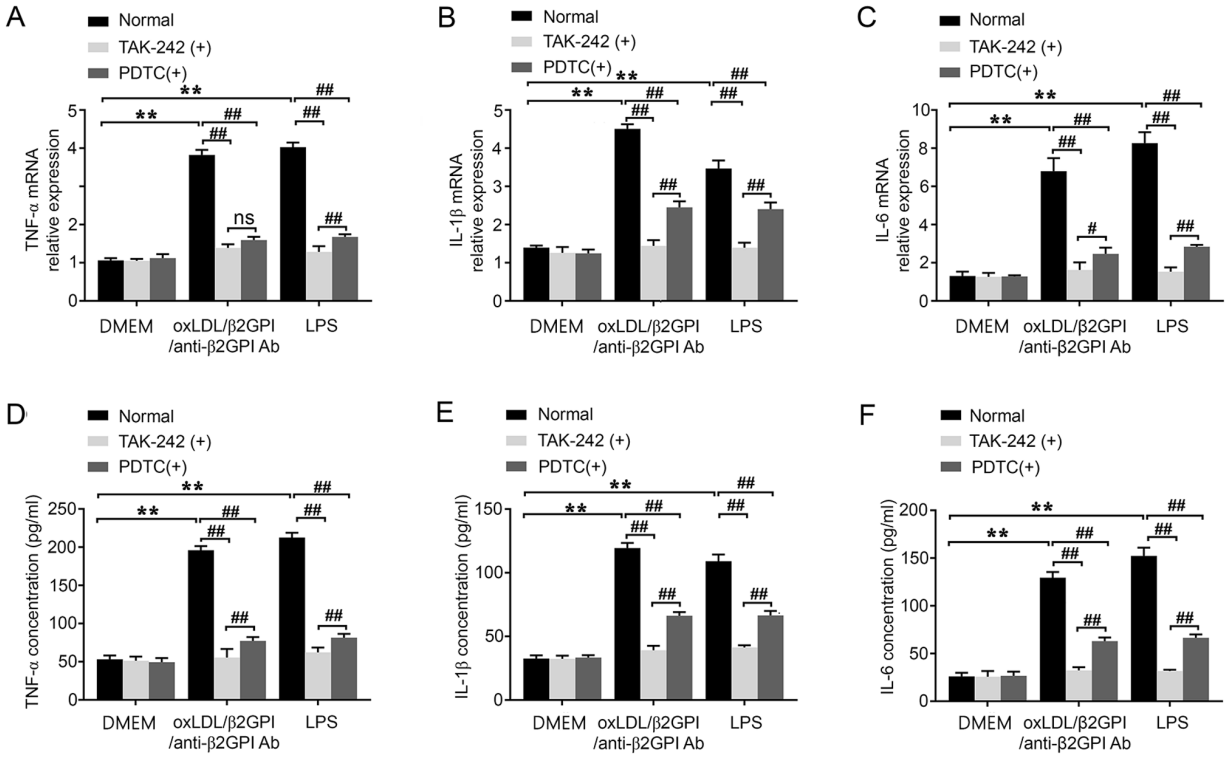

Figure 3. Effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression levels of the inflammatory cytokines, TNF-α, IL-1β and IL-6, and the involvement of the Toll-like receptor 4/NF-κB signaling pathway. HUVECs were treated with DMEM, oxLDL/β2GPI/anti-β2GPI Ab complex and LPS with or without TAK-242 or PDTC pretreatment. Total RNA was extracted from HUVECs to determine the mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 using reverse transcription-quantitative PCR. The cell culture supernatants of HUVECs were collected for analyzing the secretion of (D) TNF-α, (E) IL-1β and (F) IL-6 using commercial ELISA kits. **P<0.01; $^{\#}$P<0.05; $^{\#\#}$P<0.01. OxLDL, oxidized low-density lipoprotein; β2GPI, β2 glycoprotein I; LPS, lipopolysaccharide; HUVECs, human umbilical vein endothelial cells; ns, not significant; Ab, antibody; PDTC, 2-phosphonobutane-1,2,4,-tricarboxylic acid.

Ab complex and LPS were able to upregulate the mRNA and protein expression levels of MCP-1 compared with the DMEM control group (P<0.01; Fig. 5A and B). Similarly, the adhesion of THP-1 to HUVECs in the presence of the oxLDL/β2GPI/anti-β2GPI Ab complex or LPS was significantly increased compared with the DMEM-treated group (P<0.01; Fig. 5C and D). Moreover, these effects were significantly attenuated by the pretreatment with either TAK-242 or PDTC compared with the groups without inhibitors (P<0.01; Fig. 5). However, the attenuation effects of TAK-242 were found to be more significant than PDTC on the mRNA expression of MCP-1 induced by oxLDL/β2GPI/anti-β2GPI Ab complex or LPS, as well as the protein secretion of MCP-1 induced by LPS (P<0.01; Fig. 5A and B).

Discussion

According to the increasing basic and clinical research conducted on AS, autoimmunity has been confirmed as an important factor in its pathogenesis (5,32). An accumulating number of studies have suggested that APS, characterized by the presence of a group of heterogeneous autoantibodies and by the occurrence of thromboembolic complications in the arterial and/or venous vasculature, may be a potential cause of the accelerated progression and eventual mortality in patients with AS (33,34). Furthermore, the oxLDL/β2GPI/anti-oxLDL/β2GPI complex was identified as the circulating immune complex that exerts a proatherogenic effect in patients with APS (35). Our previous studies also discovered that the oxLDL/β2GPI/anti-β2GPI Ab complex increased the conversion of macrophages into foam cells and induced the phenotypic changes, proliferation and apoptosis of VSMCs (24,26). However, to the best of our knowledge, the roles of the oxLDL/β2GPI/anti-β2GPI Ab complex in the activation of endothelial cells have not been well-defined.

Abnormal inflammatory activation and dysfunction in the endothelium are recognized as early steps that contribute to the development and exacerbation of AS (36). The inflammatory process involves the increased expression of inflammatory cytokines, cell adhesion molecules and chemokines (37). TNF-α, which is predominantly released by endothelial cells, monocytes and macrophages during the early stages of the inflammatory response, was revealed to activate endothelial cells and macrophages and lead to the release of other inflammatory cytokines (38). In addition, IL-6 and IL-1β were found to regulate the expression of adhesion molecules on endothelial cells and enhance the recruitment of inflammatory cells to lesion sites in the early stages of the development of AS (39,40).

In the present study, HUVECs were treated with DMEM, oxLDL, oxLDL/β2GPI complex, oxLDL/anti-β2GPI Ab complex, oxLDL/β2GPI/anti-β2GPI Ab complex or LPS. LPS was confirmed to activate the vascular endothelium by affecting leukocyte adherence, and it an established natural TLR4 agonist (17). The concentrations of inflammatory cytokines (IL-1β, IL-6 and TNF-α) induced by the oxLDL/β2GPI/anti-β2GPI Ab complex and LPS were increased

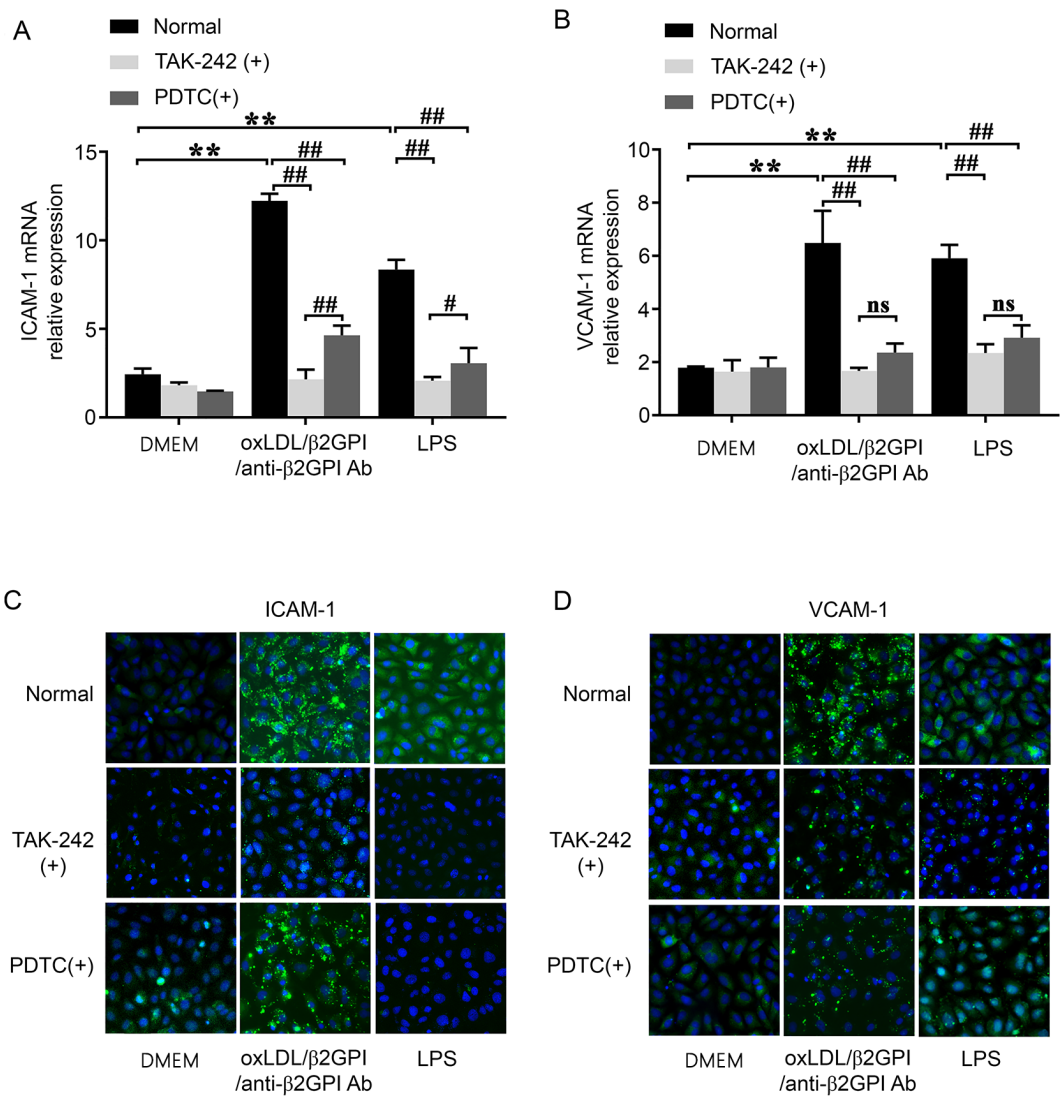

Figure 4. Effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression levels of the adhesion molecules, ICAM-1 and VCAM-1, and the involvement of the Toll-like receptor 4/NF-κB signaling pathway. HUVECs were treated with DMEM, oxLDL/β2GPI/anti-β2GPI Ab complex and LPS with or without TAK-242 or PDTC pretreatment. Total RNA was extracted from the cells to analyze the mRNA expression levels of (A) ICAM-1 and (B) VCAM-1 using reverse transcription-quantitative PCR. Representative immunofluorescence (green; magnification, x200) was used to analyze the protein expression levels of (C) ICAM-1 and (D) VCAM-1. **P<0.01; $^{\#}$P<0.05; $^{\#\#}$P<0.01. OxLDL, oxidized low-density lipoprotein; β2GPI, β2 glycoprotein I; LPS, lipopolysaccharide; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1; HUVECs, human umbilical vein endothelial cells; ns, not significant; Ab, antibody; PDTC, 2-phosphonobutane-1,2,4,-tricarboxylic acid.

compared with the DMEM group, whereas the effects of the oxLDL/β2GPI complex and oxLDL/anti-β2GPI Ab complex were relatively weaker. These findings provided evidence to support the hypothesis that the oxLDL/β2GPI/anti-β2GPI Ab complex may be able to promote the inflammatory response in endothelial cells, and that it may share a common signaling pathway with LPS. The present study also identified that the upregulated expression levels of inflammatory cytokines induced by the oxLDL/β2GPI/anti-β2GPI Ab complex could be attenuated by the inhibition of either TLR4 or NF-κB. Based on these results, it was suggested that the oxLDL/β2GPI/anti-β2GPI Ab complex may promote the endothelial inflammatory response via the TLR4-mediated intracellular signaling transduction pathway in the pathological process of AS.

TLR4-mediated intracellular signaling transduction is critical for inflammation and immune regulation, and it has been indicted to regulate the expression of genes encoding numerous important molecules involved in AS (41-43). TLR4 is expressed in different cell types present in the atherosclerotic plaque, among which it is expressed at low levels in healthy endothelial cells, but its expression is upregulated in human atherosclerotic lesions (44). The activation of the transcription factor NF-κB is known to be involved in endothelial dysfunction and the synthesis of inflammatory cytokines (45). In the present study, the activation of the TLR4/NF-κB signaling pathway during the inflammatory process was induced by the oxLDL/β2GPI/anti-β2GPI Ab complex in HUVECs. The results demonstrated that the expression of TLR4 at both the mRNA and protein levels, as well as the phosphorylation levels of NF-κB were largely increased in the presence of the oxLDL/β2GPI/anti-β2GPI Ab complex, and that the activation of NF-κB was dependent on LPS-mediated TLR4 activation. However, the effects of the oxLDL/β2GPI/anti-β2GPI complex and LPS were not completely consistent. For instance, the

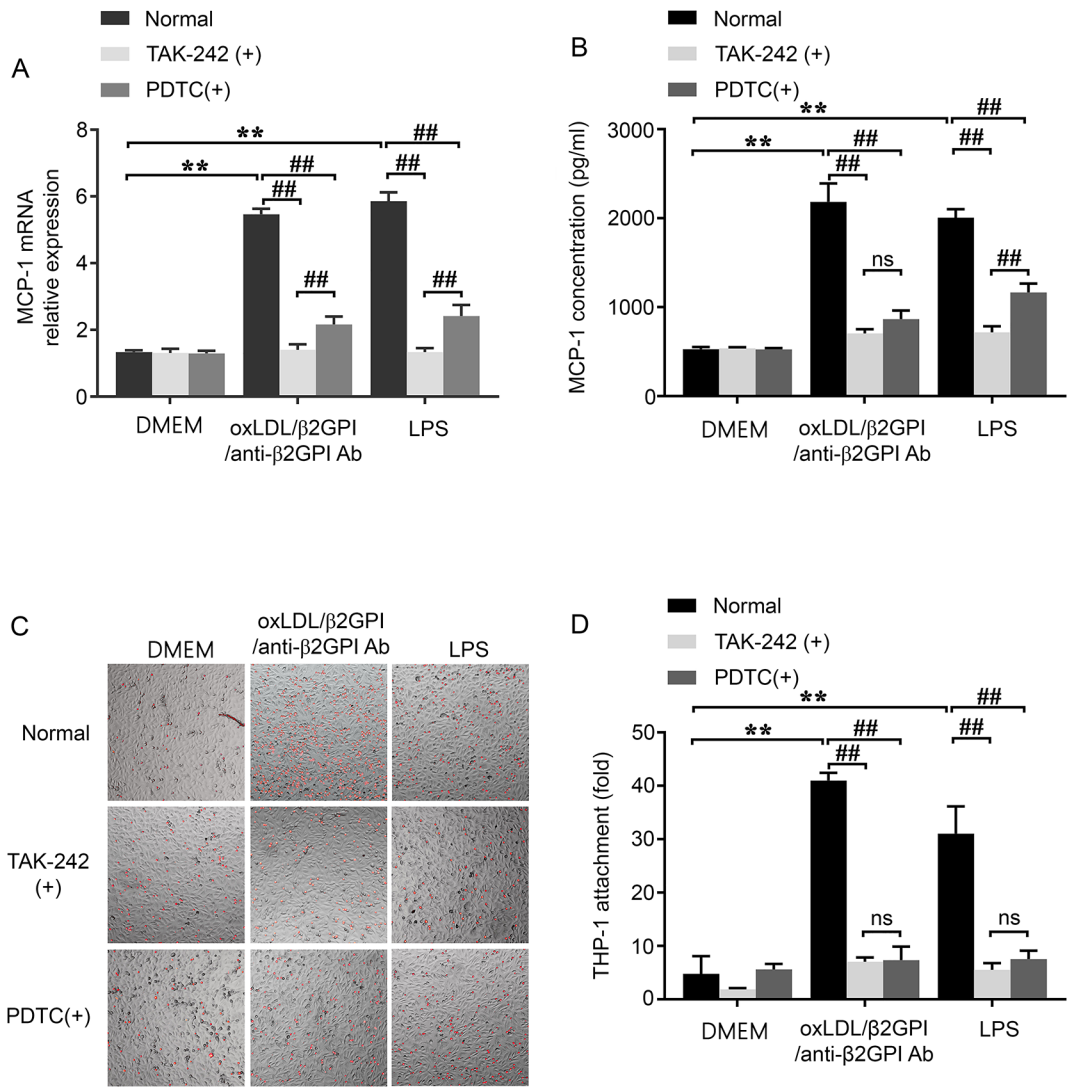

Figure 5. Effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression of MCP-1 and the adhesion of THP-1 to HUVECs, and the involvement of the Toll-like receptor 4/NF-κB signaling pathway. HUVECs were stimulated with DMEM, oxLDL/β2GPI/anti-β2GPI Ab complex or LPS pretreated with or without TAK-242 or PDTC. (A) mRNA expression levels of MCP-1 were analyzed using reverse transcription-quantitative PCR. (B) Protein expression levels of MCP-1 were detected using commercial ELISAs. (C) Monocyte recruitment ability of HUVECs were detected using a Dil-stained monocyte-EC adhesion assay (magnification, x100) and the (D) results were quantified. **P<0.01; $^{\#\#}$P<0.01. OxLDL, oxidized low-density lipoprotein; β2GPI, β2 glycoprotein I; Ab, antibody; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; ns, not significant; HUVECs, human umbilical vein endothelial cells; PDTC, 2-phosphonobutane-1,2,4,-tricarboxylic acid.

oxLDL/β2GPI/anti-β2GPI complex demonstrated weaker effects compared with LPS in inducing the expression levels of TLR4 and IL-6 and the phosphorylation of NF-κB, suggesting that TLR4-mediated NF-κB activation may be the major signaling pathway of the oxLDL/β2GPI/anti-β2GPI complex, thereby inducing the production of IL-6. By contrast, the effects of the oxLDL/β2GPI/anti-β2GPI complex in inducing the production of IL-1β, ICAM-1 and VCAM-1 were stronger compared with LPS, indicating that other AS-related cell surface receptors, such as TLR2, may also serve a role during the inflammatory activation of endothelial cells induced by the oxLDL/β2GPI/anti-β2GPI complex, in addition to TLR4.

As aforementioned, AS is characterized by vascular endothelial inflammation, and the first stage involves circulating monocytes adhering to the dysfunctional endothelium and migrating across into the sub-endothelial space, where they differentiate into macrophages (8). The concerted actions of the activated endothelial cells, monocytes and macrophages result in the production of a complex paracrine milieu of cytokines, which perpetuates a chronic proinflammatory state and fosters the progression of atherosclerotic lesion (8,46). The cell adhesion molecules, such as ICAM-1 and VCAM-1, have been identified to mediate the binding of leukocytes to vascular endothelial cells via very late activation antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1) (30,47). The present study demonstrated that the upregulated expression levels of ICAM-1 and VCAM-1 appeared to be closely associated with the oxLDL/β2GPI/anti-β2GPI Ab complex, indicating a potential central regulatory role of the oxLDL/β2GPI/anti-β2GPI Ab complex in the release of these cell adhesion molecules.

The involvement of TLR4 and NF-κB in the oxLDL/β2GPI/anti-β2GPI Ab complex-induced release of adhesion molecules was investigated in the current study

using of TAK-242 and PDTC pretreatment. TAK-242 is a novel cyclohexene that blocks the signaling mediated by the intracellular domain of TLR4 (48), while PDTC inhibits the activation of NF-κB by suppressing both NF-κB DNA binding and NF-κB-dependent transcriptional activity (49). TAK-242 and PDTC could largely attenuate the effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression levels of ICAM-1 and VCAM-1, suggesting that TLR4 and NF-κB may contributes to the signaling pathways that mediate endothelial inflammation induced by the oxLDL/β2GPI/anti-β2GPI Ab complex. Furthermore, the effects produced by TAK-242 on the induction of IL-1β and IL-6 and the protein secretion of TNF-α were found to be more significant compared with those induced by PDTC. Thus, it should be examined whether, in addition to NF-κB, other signaling pathways regulated by TLR4 serve a role in this process. The oxLDL/β2GPI/anti-β2GPI Ab complex induced the phosphorylation of AKT by HUVECs, which indicated that AKT, or other TLR4-related signaling pathways, such as p38 MAPK and ERK1/2 (50), may also be involved in the activation of endothelial cells induced by the oxLDL/β2GPI/anti-β2GPI Ab complex. These results may explain why the effects produced by TAK-242 are more significant compared with those produced by PDTC; however, the relevant mechanisms required further investigation.

Monocytes adhere and migrate into the sub-endothelial space in response to chemotactic signals, where they differentiate into macrophages and then transform into lipid-loaded foam cells (30,31). MCP-1 is a well-known chemotactic cytokine that regulates the recruitment of mononuclear inflammatory cells during the process of AS (31). As identified in the current study, both the oxLDL/β2GPI/anti-β2GPI Ab complex and LPS treatment upregulated the mRNA and protein expression levels of MCP-1. Similarly, the number of THP-1 cells bound to the HUVECs monolayer was increased by the oxLDL/β2GPI/anti-β2GPI Ab complex and LPS treatment. Notably, the effects of the oxLDL/β2GPI/anti-β2GPI Ab complex were stronger compared with LPS treatment. These results are consistent with the effects of the oxLDL/β2GPI/anti-β2GPI Ab complex on the expression of IL-1β, ICAM1 and VCAM-1. Furthermore, the effects of the oxLDL/β2GPI/anti-β2GPI Ab complex were found to be impaired by the inhibition of TLR4 or NF-κB. These results suggested that the oxLDL/β2GPI/anti-β2GPI Ab complex may promote the recruitment of monocytes to endothelial cells via the TLR4/NF-κB signaling pathway.

In conclusion, the present study demonstrated that the oxLDL/β2GPI/anti-β2GPI Ab complex significantly upregulated the expression levels of proinflammatory cytokines and pro-adhesive molecules in HUVECs, as well as enhancing their ability to recruit monocytes. Moreover, the TLR4/NF-κB signaling pathway was indicated to be involved in these processes. However, the present study has limitations. In addition to TLR4, the other endothelial cell surface receptors, including TLR2 and Fcγ, may also be involved in the oxLDL/β2GPI/anti-β2GPI complex-induced endothelial activation, should be further determined to clarify the results of the present study. Overall, the available results suggested that autoimmune factors may be potential molecular targets for the therapeutic intervention at the early vascular inflammatory stage of patients with AS with an autoimmune background. However, further studies are required to clarify the underlying mechanisms.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author's contributions

HZ and JY conceived and designed the study. GZ and QC performed the experiments. CH and YC collected the experimental results. PZ, LX and TW analyzed and interpreted the experimental data. GZ drafted and revised the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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