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Effects of β2/aβ2 on oxLDL-induced CD36 activation in THP-1 macrophages

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ABSTRACT

Aims: β2-glycoprotein I/anti-β2-glycoprotein I antibody complex (β2/aβ2) could promote oxLDL-induced endothelial inflammation through Toll-like receptor 4 (TLR4), therefore accelerates atherosclerosis in patients with anti-phospholipid syndrome (APS). However, effects of β2/aβ2 and TLR4 on oxLDL-induced CD36 activation in macrophages remain to be elucidated and are currently under investigation.

Materials and methods: THP-1 macrophages with or without the pre-treatment of TAK-242, a TLR4 inhibitor, were treated with RPMI 1640, oxLDL, oxLDL + β2/aβ2 or oxLDL + LPS.CD36 expression and subsequent intracellular lipid accumulation, cholesterol-transportation-related proteins (ACAT1, ABCG1 and ABCA1) expression, inflammatory cytokines (IL-1β, TNF-α and IL-6) secretion, focal adhesion kinases (FAK) activation and matrix metalloproteinases (MMP-2 and MMP-9) expression by these THP-1 macrophages were evaluated. Moreover, effects of TLR4 on oxLDL + β2/aβ2-induced peroxisome proliferators-activated receptor-γ (PPAR-γ) expression and CD36 translocation have also been observed.

Key findings: Compared with oxLDL-treated ones, CD36 expression, intracellular lipid accumulation and FAK activation were inhibited, whereas the levels of inflammatory cytokines and MMPs were upregulated in THP-1 macrophages treated with oxLDL + β2/aβ2 ($p < 0.05$). Moreover, observed differences between oxLDL-treated and oxLDL + β2/aβ2-treated THP-1 macrophages could be reversed by TAK-242 pre-treatment ($p < 0.05$). Furthermore, oxLDL + β2/aβ2 promoted PPAR-γ expression and CD36 cytoplasmic translocation in THP-1 macrophages, these effects could also be attenuated by TAK-242 ($p < 0.05$).

Significance: Through a TLR4 dependent manner, β2/aβ2 inhibited oxLDL-induced CD36 expression, lipid accumulation and FAK activation, while promoted inflammatory cytokines and MMPs expression in THP-1 macrophages, indicating the novel dual roles played by β2/aβ2 in APS-related atherosclerosis.

1. Introduction

Oxidized low density lipoprotein (oxLDL) plays a vital role in atherosclerosis (AS) by promoting vascular inflammation and monocytes recruitment, which then accelerates atherosclerotic plaque formation [1,2]. CD36 is the premier receptor for subendothelial oxLDL which recognizes and engulfs oxLDL, and mediates intracellular lipid accumulation by regulating the expression of cholesterol-transportation-related proteins including acyl coenzyme A cholesterol acyl transfer enzyme1 (ACAT1), ATP-binding cassette transporter G-1 (ABCG1) and ATP-binding cassette transporter A-1 (ABCA1) [3–5]. On the other hand, CD36 upregulates focal adhesion kinases (FAK) expression and phosphorylation, and thus promotes matrix metalloproteinases (MMPs) expression which facilitates macrophages retention in the subendothelial space [6–8]. Besides, CD36 also promotes inflammatory cytokines secretion by macrophages when it serves as the co-receptor of Toll-like receptors (TLRs) [9–11].

Patients with antiphospholipid syndrome (APS) are often accompanied by atherosclerotic changes, such as endothelial inflammation and intimal thickening [12]. This was partly blamed for β2-glycoprotein I (β2GPI) which was accepted as the key autoantigen in APS. β2GPI interacts with its autoantibody to form a β2GPI/anti-β2GPI antibody complex (β2/aβ2), and then triggers inflammatory response and proliferation in atherosclerotic cells including endothelial cells (ECs), smooth muscle cells (SMCs) and monocytes/macrophages, via Toll-like receptor 4 (TLR4) signaling pathway [13,14]. However, although APS patients were once considered to be prone to atherosclerosis because of the excessive inflammatory response, the overall incidence of AS in these patients did not increase significantly [15,16].

Here, we used oxLDL as the basic stimuli, and examined the effects of β2/aβ2 and TLR4 on oxLDL-induced pro-atherosclerotic changes in THP-1 macrophages. Our results revealed that β2/aβ2 promotes

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cytokines secretion and FAK activation in THP-1 macrophages, but unexpectedly inhibited CD36 expression and intracellular lipid accumulation in a TLR4 dependent manner.

2. Materials and methods

2.1. Induction of THP-1 macrophages

THP-1, a human monocytes cell-line, was purchased from the American Type Culture Collection (ATCC, USA). THP-1 monocytes were cultured at 37 °C in a 5% CO_2/95% humidified air incubator (ThermoFisher, USA) in RPMI 1640 (BI, Israel) with 10% FBS (BI, Israel) and 1% penicillin-streptomycin antibiotic (Gibico, USA). We added 100 ng/ml 2-Acetoxy-1-methoxypropane (PMA) (Enzo, Sweden) to THP-1 monocytes for 48 h to induce THP-1 macrophages. Then the induced THP-1 macrophages were identified by their morphological features and surface marker expression.

2.2. Preparation of stimuli

Stimuli in current study were oxLDL (50 μg/ml) (Yiyuan biotech, China), oxLDL (50 μg/ml) plus β2/aβ2 (200 μg/ml) (oxLDL + β2/aβ2), and oxLDL (50 μg/ml) plus lipopolysaccharide (LPS) (500 ng/ml) (Sigma-Aldrich, USA) (oxLDL + LPS).

For preparation of β2/aβ2, 100 μg β2GPI and 100 μg anti-β2GPI antibody were added to 1 ml RPMI 1640 (with 10% FBS and 1% antibiotic), and then incubated at 37 °C for 30 min. Concentrations of reagents were determined by pre-experiment or our previous studies. All reagents were diluted in RPMI 1640 (with 10% FBS and 1% antibiotic), and then tested by the limulus test to exclude the influence of exogenous LPS. Therefore, RPMI 1640 (with 10% FBS and 1% antibiotic) was employed as the blank control in the current study.

2.3. LPS and TAK-242

LPS is a TLR4 agonist that activates TLR4 signaling by binding to TLR4/CD14 complex on cell membrane [17]. TAK-242 (Invivogen, USA) could selectively bind to TLR4 and interfere its interactions with agonist, such as LPS [18]. TLR4 function in THP-1 macrophages was blocked by TAK-242 pre-treatment (1 μg/ml, 6 h before stimulation) in current study.

2.4. Oil red O staining

THP-1 macrophages were seeded in 96 well plates. After 48 h incubation with different stimuli, THP-1 macrophages were fixed with fresh 4% formalin for 30 min, and then stained by oil red O for intracellular lipids for 15 min. After washing with 60% isopropyl alcohol, nuclei of THP-1 macrophages were stained by hematoxylin for 2 min, then observed and photographed under an optical microscope (Olympus, Japan).

Intracellular oil red O was extracted by 100% isopropyl alcohol, then optical absorbance of extracting solution at 520 nm was determined by Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek, USA).

2.5. Quantitative RT-PCR (qPCR)

Total RNA samples were isolated from THP-1 macrophages by TRIzol reagent (Invitrogen, USA), then reversed transcription was carried out with 500 ng of total RNA in a 10 μL system. Expressions of target genes were analyzed by qPCR using SYBR Green I dye (Vazyme, China).

Sequence of primers for qPCR:

β-actin primers:	5′-CACGAAACTACCTTCAACTCC-3' (forward)
	5′-CATACTCCTGCTTGCTGATC′-3' (reverse)
CD36 primers:	5′-CCCAGTAGCTGCCCTATTCA-3' (forward)
	5′-ATTTGGTGCAATGAAATGC-3' (reverse)
ACAT1 primers:	5′-TATTGCCCTCCTCATTCTC-3' (forward)
	5′-CTTCATAGCGACATAACCC-3' (reverse)
ABCG1 primers primepriprimers:	5′-GGTTCTTCGTCAGCTTCGAC-3' (forward)
	5′-GTTTCCTGGCATTCAGGTGT-3' (reverse)
ABCA1 primers:	5′-TATGAGGGCCAGATCACCTC-3' (forward)
	5′-GCTGGCTTGTTTTGCTTTTC-3' (reverse)
TNF-α primers:	5′-CCTCTCTCTAATCAGCCCTCTG-3' (forward)
	5′-GAGGACCTGGGAGTAGATGAG-3' (reverse)
IL-1β primers:	5′-TCGCCAGTGAAATGATGGCT-3' (forward)
	5′-GGTCGGAGATTCGTAGCTGG-3' (reverse)
FAK primers:	5′-TGGTGCAATGGAGCGAGTATT-3' (forward)
	5′-CAGTGAACCTCCTCTGACCG-3' (reverse)
IL-6 primers:	5′-TGCAATAACCACCCCTGACC-3' (forward)
	5′-ATTTGCCGAAGAGCCCTCAG-3' (reverse)

Relative mRNA expressions of target genes compared to β-actin were calculated by $2^{-\Delta\Delta CT}$ method.

2.6. Immunoblot

THP-1 macrophages were lysed in RIPA buffer (Biyotime, China) supplemented with 0.5% protease inhibitors (Biyotime, China) and 1% phosphatase inhibitors (Wanlei biotech, China). Size-separated proteins were transferred to PVDF membranes (Millipore, Germany), which then were incubated with primary antibodies specific for β-actin (abcam, UK), CD36 (abcam, UK), FAK (t-FAK) (abcam, UK), phosphorylated FAK (pFAK) (Cell signaling technology, USA) and PPAR-γ (Wanlei biotech, China). After washing the membranes, they were incubated with secondary antibodies that were conjugated with horseradish peroxidase. Blots were then prepared for enhanced chemiluminescence and subsequent autoradiography. The protein concentration was determined using a BCA protein assay kit (ThermoFisher, USA).

2.7. Immunofluorescence staining

THP-1 macrophages were seeded in 24 well plates. After 24 h incubation with stimuli, THP-1 macrophages were fixed in 4% paraformaldehyde for 30 min. After washing with PBS for three times, THP-1 macrophages were blocked with 5% BSA at 37 °C for 1 h, and then incubated overnight at 4 °C with primary antibodies specific for CD36 and FAK (abcam, UK). After washing with PBS, THP-1 macrophages were incubated with AF488-conjugated secondary antibody (FcMACS, China) or RBTIC-conjugated secondary antibody (Biyotime, China), and then observed and imaged by Cytation™ 5 Cell Imaging Multi-Mode Reader (Bio Tek, USA).

2.8. Cytokine assay

THP-1 macrophages were seeded in 96 well plates. After 48 h incubation with stimuli, culture supernatant of THP-1 macrophages were collected. Then protein levels of TNF-α, IL-1β, IL-6, MMP-2 and MMP-9 in the culture supernatant were measured by Sunny ELISA kits (Multiscience, China), and data was calculated by ELISA Calc software.

2.9. Statistical analysis

All experiments were replicated a minimum of 3 times. Normally distributed variables were expressed as means ± SD. Differences between control and experimental conditions were assessed using the one-way ANOVA. Two-factor treatment results were analyzed by two-way ANOVA. All statistical analyses were performed using IBM SPSS Software version 22.0.0.0. Statistical significance was defined as $p < 0.05$.

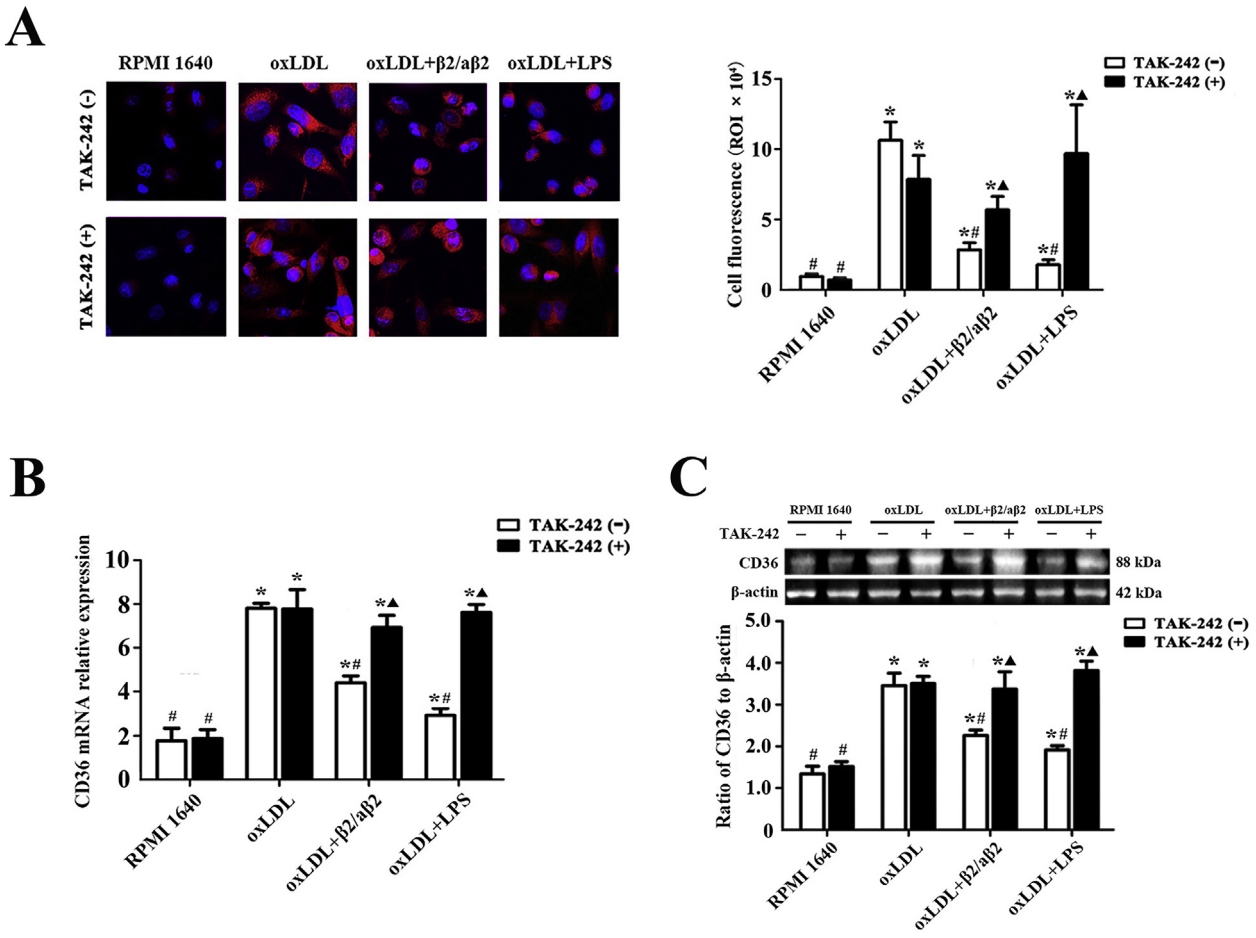


Fig. 1. Effects of β2/aβ2 and TLR4 on oxLDL-induced CD36 expression by THP-1 macrophages

(A) Representative immunofluorescence images of THP-1 macrophages stained for CD36 protein (Magnification: × 200). RBITC-labeled secondary antibody was shown in red and the nuclei stained with DAPI was in blue. The height of each bar on the right panel indicates the intense of red fluorescence in THP-1 macrophages. (B) Quantitative PCR analysis of CD36 mRNA relative expression by THP-1 macrophages. The numbers on the y-axis represent fold difference in specific gene expression. (C) Immunoblot analysis for CD36 protein in the lysates from THP-1 macrophages. The numbers on the y-axis of histogram represent fold difference in CD36 protein expression. $^{*}p < 0.05$ vs RPMI 1640 group, $^{\#}p < 0.05$ vs oxLDL group, $^{\blacktriangle}p < 0.05$ vs corresponding TLR4-inhibited group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Effects of β2/aβ2 and TLR4 on oxLDL-induced CD36 expression by THP-1 macrophages

We cultured THP-1 macrophages (Fig. S1) and examined their CD36 expression. Detected by cellular immunofluorescence, β2/aβ2 partly attenuated oxLDL-induced CD36 expression (red fluorescence) by THP-1 macrophages ($p < 0.05$) (Fig. 1A). β2/aβ2 could active TLR4 in many cell types including macrophages, thus we have noted the elevated TLR4 level in β2/aβ2-treated THP-1 macrophages ($p < 0.05$) (Fig. S2). Because of this, we used LPS and TAK-242 to unveil the role played by TLR4 in oxLDL-induced CD36 expression. Similar to β2/aβ2, LPS could down-regulate oxLDL-induced CD36 level in THP-1 macrophages. Moreover, TAK-242 pre-treatment did not affect CD36 level in oxLDL-treated THP-1 macrophages, but attenuated the negative regulatory effects of β2/aβ2 and LPS on oxLDL-induced CD36 expression ($p < 0.05$) (Fig. 1A).

We further confirmed our results by using qPCR and immunoblot analysis, and found that mRNA and protein levels of CD36 in oxLDL + β2/aβ2-treated THP-1 macrophages were lower than in oxLDL-treated macrophages, but still significantly higher than in RMPI 1640-treated ones. Moreover, the suppressive effects of β2/aβ2 on oxLDL-induced CD36 expression were completely restored by TAK-242 ($p < 0.05$) (Fig. 1B–C).

3.2. Effects of β2/aβ2 and TLR4 on oxLDL-induced lipid accumulation in THP-1 macrophages

We performed oil red O stain to visualize the intracellular lipid in THP-1 macrophages. The density of red-stained particles and OD 520 nm of extracting solution was higher in THP-1 macrophages treated with oxLDL + β2/aβ2 or oxLDL + LPS. In addition, the suppressive effects of β2/aβ2 and LPS on oxLDL-induced lipid accumulation in THP-1 macrophages were completely and partly eliminated by TAK-242, separately ($p < 0.05$) (Fig. 2A).

ACAT1 played an indispensable role in lipid influx and accumulation, whereas ABCG1 and ABCA1 are effective in lipid efflux [4,19,20]. We found that ABCA1 and ABGC1 levels in THP-1 macrophages were separately regulated upwards and downwards after oxLDL stimulation. Moreover, β2/aβ2 and LPS attenuated the effects of oxLDL on ACAT1 and ABCG1 expression, whereas their function could be weakened by TAK-242 ($p < 0.05$) (Fig. 2B–C). We also surprisingly found that ABCA1 level was not influenced by oxLDL or other stimuli, but upregulated by TAK-242 ($p < 0.05$) (Fig. 2D).

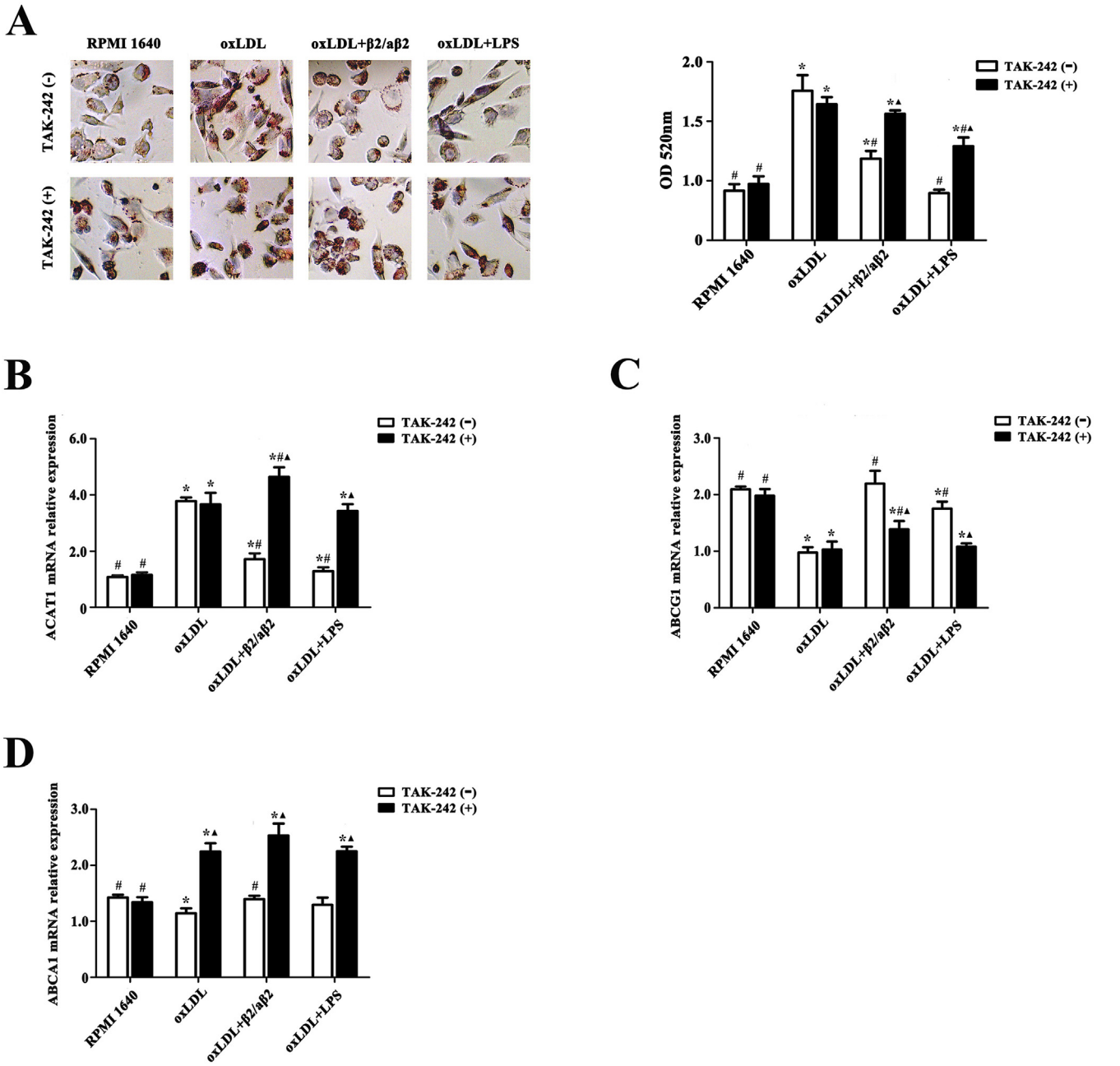


Fig. 2. Effects of β2/aβ2 and TLR4 on oxLDL-induced lipid accumulation in THP-1 macrophages

(A) Representative images of THP-1 macrophages stained for intracellular lipids by oil red O (Magnification: × 200). Oil red O-stained lipid was shown in red and the nuclei counterstain with hematoxylin was in blue. The height of each bar on the right panel indicates the optical density of extracting solution. (B) qPCR analysis of ACAT1 mRNA relative expression by THP-1 macrophages. (C) qPCR analysis of ABCG1 mRNA relative expression by THP-1 macrophages. (D) qPCR analysis of ABCA1 mRNA relative expression by THP-1 macrophages. The numbers on the y-axis represent fold difference in specific gene expression. $^{*}p < 0.05$ vs RPMI 1640 group, $^{\#}p < 0.05$ vs oxLDL group, $^{\blacktriangle}p < 0.05$ vs corresponding TLR4-inhibited group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Effects of β2/aβ2 and TLR4 on oxLDL-induced TNF-α, IL-1β and IL-6 expression by THP-1 macrophages

Total RNA and culture supernatant were harvested from THP-1 macrophages for the detection of inflammatory cytokines, including TNF-α, IL-1β and IL-6. Upregulated mRNA levels of TNF-α, IL-1β and IL-6 were noted in THP-1 macrophages treated with oxLDL, and was even higher in ones treated with oxLDL + β2/aβ2 or oxLDL + LPS ($p < 0.05$) (Fig. 3A, C, E). Moreover, protein levels of TNF-α, IL-1β and IL-6 in culture supernatant were in consistent with their gene levels ($p < 0.05$) (Fig. 3C–D).

Then we examined whether TAK-242 pre-treatment could inhibit the capacity of oxLDL, oxLDL + β2/aβ2 and oxLDL + LPS to induce TNF-α, IL-1β and IL-6 expression by THP-1 macrophages. In line with our expectations, the suppressive effects of β2/aβ2 and LPS on oxLDL-induced TNF-α, IL-1β and IL-6 expression were attenuated by TAK-242. Moreover, TNF-α and IL-6 levels in THP-1 macrophages treated with oxLDL was down-regulated by TAK-242, while IL-1β level did not ($p < 0.05$) (Fig. 3).

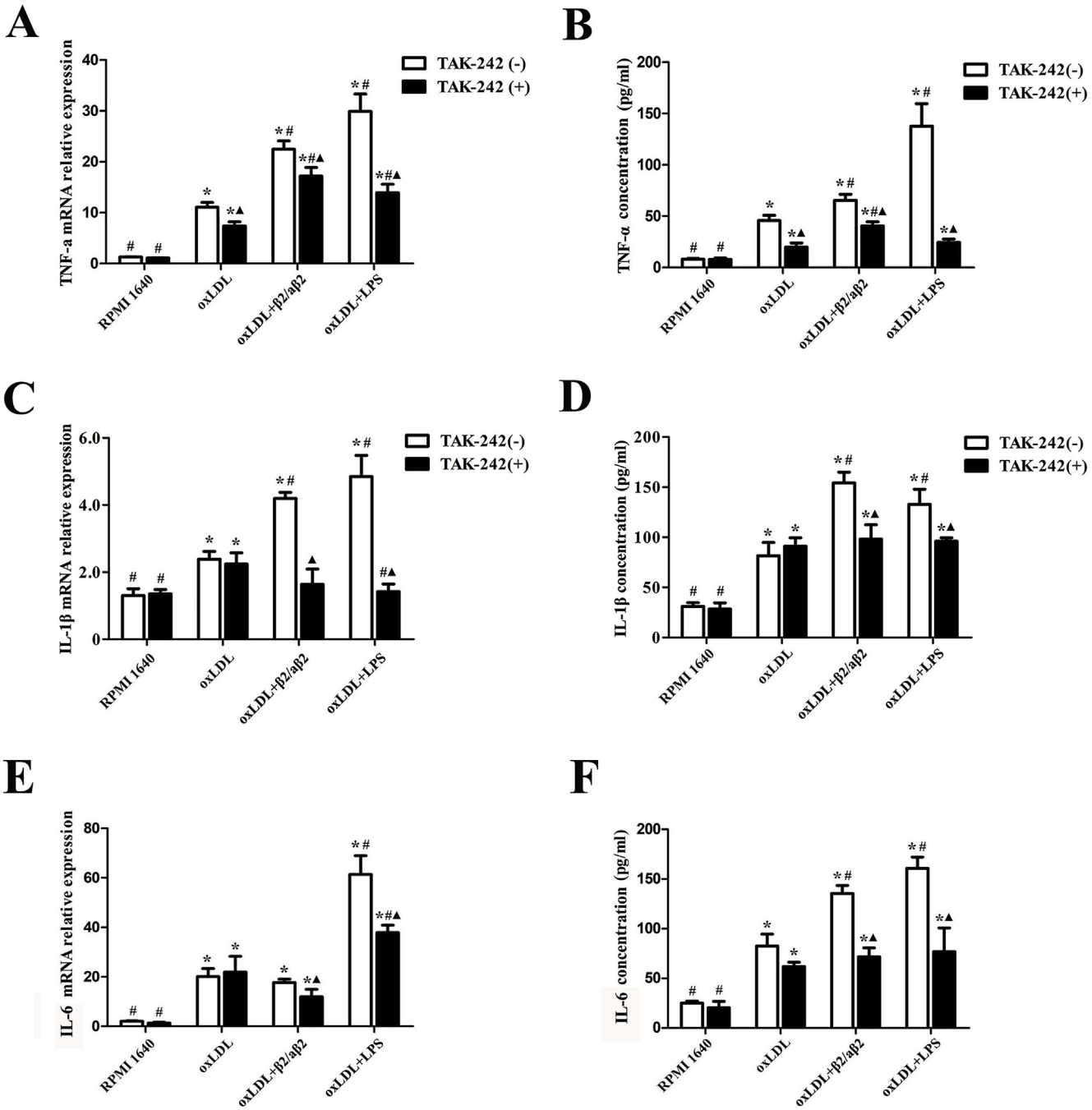


Fig. 3. Effects of β2/aβ2 and TLR4 on oxLDL-induced TNF-α, IL-1β and IL-6 expression by THP-1 macrophages

(A) qPCR analyses of TNF-α mRNA relative expression by THP-1 macrophages. (B) ELISA analyses of TNF-α protein in culture supernatant of THP-1 macrophages. (C) qPCR analyses of IL-1β mRNA relative expression by THP-1 macrophages. (D) ELISA analyses of IL-1β protein in culture supernatant of THP-1 macrophages. (E) qPCR analyses of IL-6 mRNA relative expression by THP-1 macrophages. (F) ELISA analyses of IL-6 protein in culture supernatant of THP-1 macrophages. The numbers on the y-axis of panel A, C and E represent fold difference in specific gene expression. $^{*}p < 0.05$ vs RPMI 1640 group, $^{\#}p < 0.05$ vs oxLDL group, $^{\blacktriangle}p < 0.05$ vs corresponding TLR4-inhibited group.

3.4. Effects of β2/aβ2 and TLR4 on oxLDL-induced FAK activation in THP-1 macrophages

As a down-stream molecule of CD36, FAK contributes to AS development by promoting macrophages subendothelial retention and MMPs-induced ECM (extracellular matrix) degradation [6,21]. We found that β2/aβ2 and LPS could partly reverse oxLDL-induced FAK protein expression (green fluorescence) by THP-1 macrophages ($p < 0.05$) (Fig. 4A). The effects of β2/aβ2 and LPS on oxLDL-induced FAK expression were further confirmed on gene level by qPCR detection ($p < 0.05$) (Fig. 4B). We also found that oxLDL-induced FAK phosphorylation (at Tyr397) was suppressed by β2/aβ2 and LPS ($p < 0.05$) (Fig. 4C). Moreover, negative regulatory effects of β2/aβ2 and LPS on oxLDL-induced FAK expression and phosphorylation could be completely blocked by application of TLR4 inhibitor, TAK-242 ($p < 0.05$) (Fig. 4A–C).

We also examined MMP-2 and MMP-9 secretion by THP-1 macrophages, which we assessed their concentration in culture supernatant by ELISA. THP-1 macrophages treated with oxLDL secreted less MMP-2 and MMP-9 than did ones treated with oxLDL + β2/aβ2 or

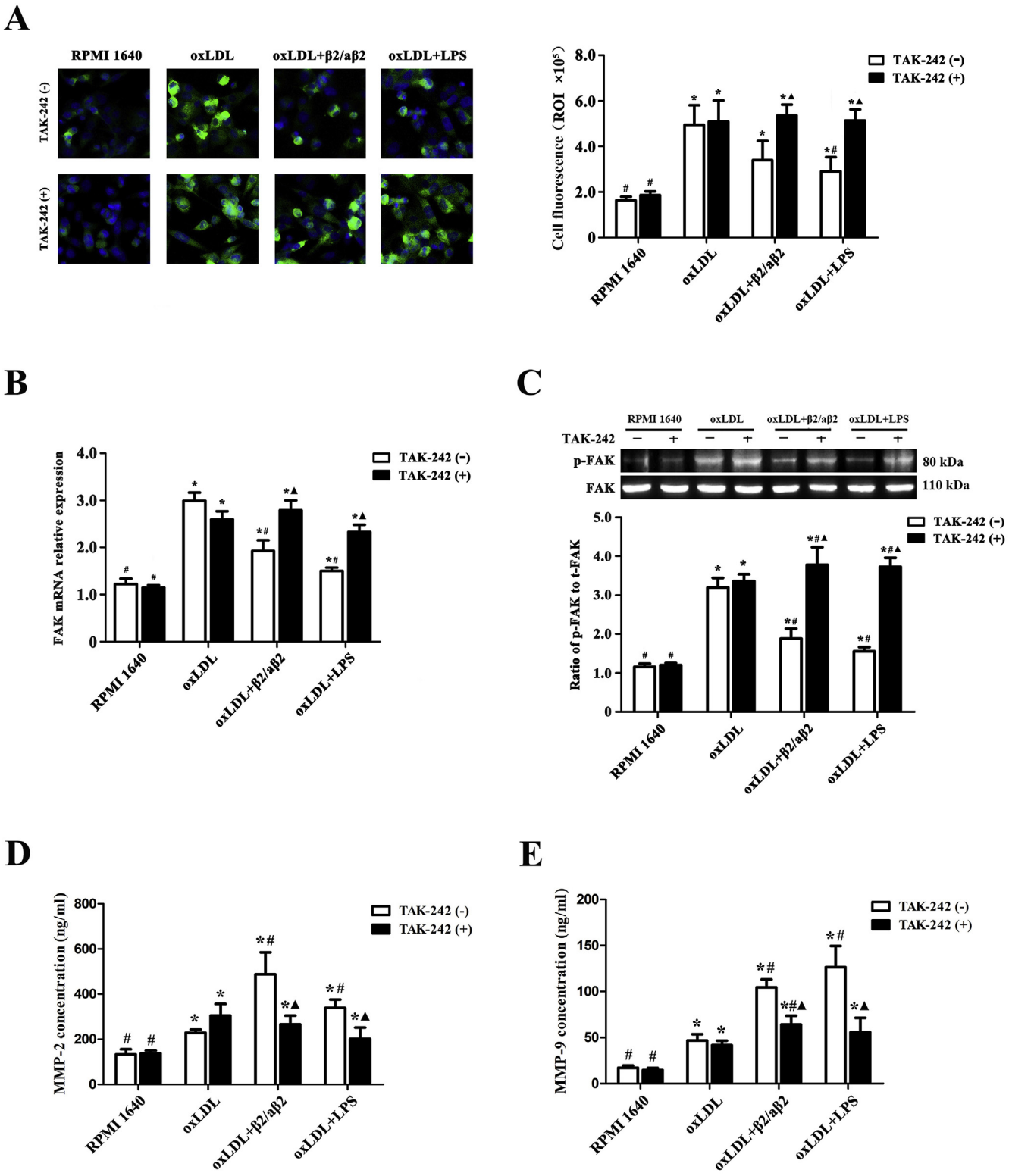
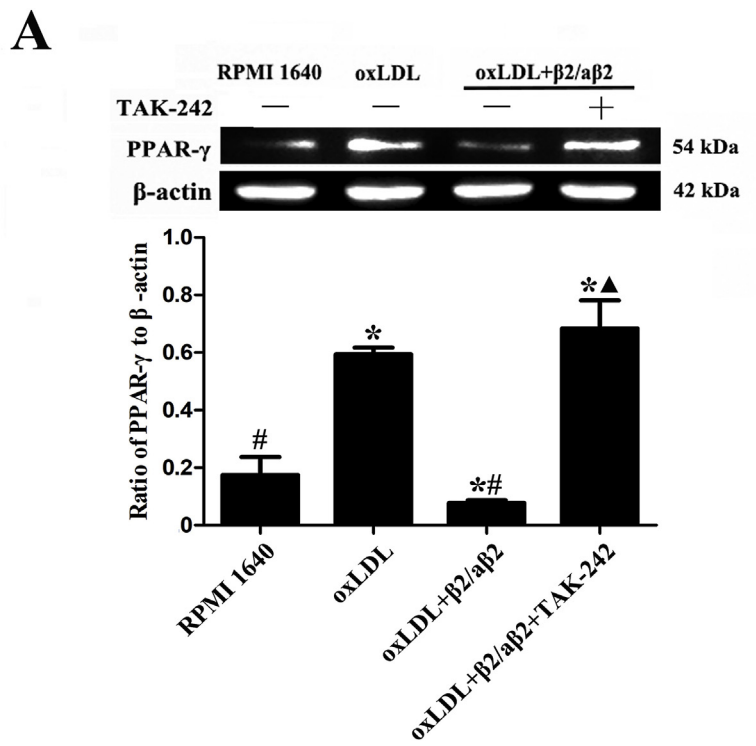


Fig. 4. Effects of β2/aβ2 and TLR4 on oxLDL-induced FAK activation in THP-1 macrophages

(A) Representative immunofluorescence images of THP-1 macrophages stained for FAK protein (Magnification: × 200). AF488-labeled secondary antibody was shown in green and the nuclei stained with DAPI was in blue. The height of each bar on the right panel indicates the intense of green fluorescence in THP-1 macrophages. (B) qPCR analysis of FAK mRNA relative expression by THP-1 macrophages. The numbers on the y-axis represent fold difference in FAK gene expression. (C) Immunoblot analysis for p-FAK protein in the lysates from THP-1 macrophages. The numbers on the y-axis of histogram represent fold difference in p-FAK protein expression. (D) ELISA analysis of MMP-2 protein in culture supernatant of THP-1 macrophages. (E) ELISA analysis of MMP-9 protein in culture supernatant of THP-1 macrophages. $^{*}p < 0.05$ vs RPMI 1640 group, $^{\#}p < 0.05$ vs oxLDL group, $^{\blacktriangle}p < 0.05$ vs corresponding TLR4-inhibited group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



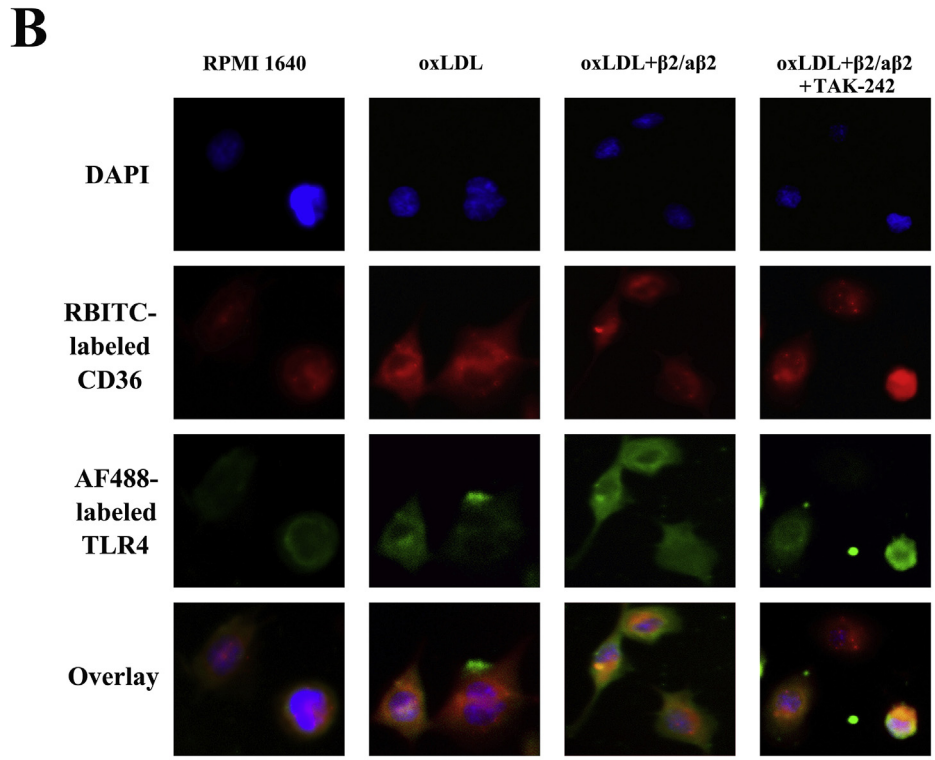


Fig. 5. Effects of β2/aβ2 and TLR4 on oxLDL-induced PPAR-γ expression and CD36 subcellular location in THP-1 macrophages

(A) Immunoblot analysis for PPAR-γ protein in the lysates from THP-1 macrophages. The numbers on the y-axis of histogram represent fold difference in PPAR-γ protein expression. (B) Representative immunofluorescence images of THP-1 macrophages stained for CD36 and TLR4 protein (Magnification: × 200). Cell-conjugated TLR4 and CD36 primary antibodies were detected by AF488-labeled and RBTIC-labeled secondary antibodies separately. TLR4 protein was shown in green, CD36 protein was in red, and the nuclei stained with DAPI was in blue. $^{*}p < 0.05$ vs RPMI 1640 group, $^{\#}p < 0.05$ vs oxLDL group, $^{\blacktriangle}p < 0.05$ vs corresponding TLR4-inhibited group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

oxLDL + LPS. Moreover, the differences in MMP-2 and MMP-9 secretion between oxLDL-treated and oxLDL + β2/aβ2-treated or oxLDL + LPS-treated THP-1 macrophages were reduced by TAK-242 ($p < 0.05$) (Fig. 4D–E).

3.5. Effects of β2/aβ2 and TLR4 on oxLDL-induced PPAR-γ expression and CD36 subcellular location in THP-1 macrophages

We further examined the level of PPAR-γ that could promote CD36 expression in THP-1 macrophages. Compared with RMPI 1640, PPAR-γ expression by THP-1 macrophages was promoted by oxLDL, whereas inhibited by oxLDL + β2/aβ2. When TLR4 function was blocked by TAK-242, the suppressive effect of β2/aβ2 on oxLDL-induced PPAR-γ expression was completely reversed ($p < 0.05$) (Fig. 5A).

Cellular immunofluorescence was used to observe CD36 subcellular localization. A prior study found that TLR4 was strictly localized to the cell membrane in macrophages [22]. Thus, TLR4 protein (green fluorescence) was used as the marker of cell membrane in current study. We

found that CD36 (red fluorescence) co-localized with TLR4 in THP-1 macrophages treated with oxLDL but not oxLDL + β2/aβ2. Moreover, when pre-treated with TAK-242, CD36 protein and TLR4 protein were co-localized on the membrane in THP-1 macrophages treated with oxLDL + β2/aβ2. These results indicated that β2/aβ2 could induce CD36 translocation from cell membrane to cytoplasm in a TLR4-dependent manner, as such inhibits CD36-induced oxLDL endocytosis (Fig. 5B).

Furthermore, we also note that TLR4 protein expression was upregulated in THP-1 macrophages treated with oxLDL + β2/aβ2, which could be inhibited by TAK-242 (Fig. 5B). These results were in consistent with those we obtained from qPCR and immunoblot analysis ($p < 0.05$) (Fig. S2).

4. Discussion

Here we presented data demonstrating that β2/aβ2 played a regulatory role in oxLDL-induced CD36 cell-surface expression and subsequent intracellular lipid accumulation, inflammation and FAK activation in THP-1 macrophages. As the main immune complex in APS patients, β2/aβ2 notably inhibited the oxLDL-induced CD36 expression by THP-1 macrophages. However, when THP-1 macrophages were pretreated with a TLR4 inhibitor, TAK-242, the regulatory effect of this immune complex on CD36 expression was almost eliminated (Fig. 1). These results were corroborated by the data of CD36 gene expression on LPS-stimulated monocytes published by Zamora et al. [23]. However , the results from Hashimoto et al. showed that LPS enhances CD36 expression and acetylated-LDL uptake by bone marrow (BM) macrophages [24]. The different modifications of LDL and the interaction between BM macrophages and other BM stromal cells should be responsible for the differences in results between Hashimoto's and our reports.

In a previous report, we observed a promoted lipid accumulation in oxLDL/β2GPI/anti-β2GPI-treated mouse peritoneal macrophages [25]. In that study, β2GPI was pre-treated with oxLDL to form a stable oxLDL/β2GPI complex, then peritoneal macrophages were exposed to the oxLDL/β2GPI complex and anti-β2GPI antibody. However, high level of β2/aβ2 were detectable in the serum of APS patients, whereas oxLDL mainly exist in the vascular wall but not in peripheral blood. Moreover, we also noted that either β2GPI or anti-β2GPI stimulation promoted CD36 mRNA level in THP-1 macrophages, but β2/aβ2-only induced an intermediate level of CD36 mRNA expression, which strongly indicated the interaction between β2GPI and anti-β2GPI (Fig. S3). In this case, it is important to evaluate the effects of β2/aβ2 on macrophages activation.

Oil red O stain and Trinder assay were used to evaluate intracellular lipid accumulation in THP-1 macrophages. Number of oil red O positive stained particles and the optical density of extracting solution were less in oxLDL + β2/aβ2-treated THP-1 macrophages than in oxLDL-treated ones (Fig. 2A). According to our results (Table S1), CE proportion in oxLDL + β2/aβ2-treated THP-1 macrophages (51.1%) was lower than that in oxLDL-treated cells (58.2%), though both of them meet the criterion of foam cell (> 50%). Prior studies found that lipid-phagocytic function of macrophages (including resident macrophages such as microglia) was negatively regulated by TLR4 [26,27]. We found that β2/aβ2 promoted TLR4 expression by THP-1 macrophages (Fig. S2). Moreover, when TLR4 function was blocked by TAK-242, the difference in intracellular lipid levels between oxLDL + β2/aβ2-treated and oxLDL-treated THP-1 macrophages became less apparent (Fig. 2A, Table S1).

One study found that TLR4-regulated lipid metabolism in mice depends on the activation of ABCA1-mediated lipid efflux [28]. However, the oxLDL-induced ABCA1 gene expression in the current study was significantly influenced by TAK-242 pre-treatment but not β2/aβ2, marking it less unlikely that the TLR4-dependent effects of β2/aβ2 on macrophages lipid accumulation was related to ABCA1 (Fig. 2D). Moreover, the level of IL-6, a cytokine that negatively regulates ABCA1 expression [23,29], was significantly decreased in TAK-242 pre-treated macrophages (Fig. 3E–F). Conceivably, the upregulated ABCA1 level, which was induced by TAK-242 pre-treatment, is more likely a consequence of suppressed IL-6 secretion.

Instead of ABCA1, current study highlights the importance of ABCG1 and ACAT1 in THP-1 macrophages lipid accumulation. Like ABCA1, ABCG1 mediates the efflux of intracellular lipid and thus antagonizes the effects of ACAT1, the only known cholesterol esterase in human body [30,31]. We found that ABCG1 mRNA level was higher, while ACAT1 mRNA level was lower in oxLDL + β2/aβ2-treated THP-1 macrophages in comparation with oxLDL-treated ones. We also found TAK-242 pre-treatment could reverse the differences of ABCG1 and ACAT1 mRNA expression between oxLDL + β2/aβ2-treated and oxLDL-treated THP-1 macrophages (Fig. 2B–C). Together, β2/aβ2 inhibits oxLDL-induced intracellular lipid accumulation by regulating ACAT1-mediated cholesterol esterification and ABCG1-mediated cholesterol efflux, which appears associated with the function of TLR4.

Expression of TNF-α, IL-1β and IL-6 were used to evaluate the inflammatory response in THP-1 macrophages. We found that oxLDL + β2/aβ2-induced higher levels of TNF-α, IL-1β and IL-6 than did oxLDL. Then we evaluate the influence of TLR4 on inflammatory cytokines secretion. When TLR4 function was impaired by TAK-242 pretreatment, the β2/aβ2-induced high expression of TNF-α, IL-1β and IL-6 were partly or completely reversed (Fig. 3). Indeed, these results demonstrated that β2/aβ2 and TLR4 facilitate oxLDL-induced inflammation in THP-1 macrophages, which were in line with the results of studies on monocytes, ECs and SMCs [14,32].

Prior studies found that CD36 activation could induce the expression and phosphorylation (at Tyr397) of FAK, and promote the secretion of MMP-2 and MMP-9 in macrophages [23,33,34]. We found that FAK in THP-1 macrophages was activated by oxLDL, which was characterized by up-regulated FAK expression and phosphorylation. Moreover, β2/aβ2 negatively regulated the oxLDL-induced FAK activation, whose effects could be attenuated by TAK-242 pre-treatment (Fig. 4A–C).

A previous report from Wang et al. demonstrated that anti-β2GPI antibody could promote MMP-9 expression in the aortic arch of high-fat diet mice [35]. Similar results were obtained in oxLDL + β2/aβ2-treated THP-1 macrophages in the current study. We found that oxLDL + β2/aβ2 induced higher expression of MMP-2 and MMP-9 than did oxLDL, and the difference between them could be reduced by TAK-242 pretreatment (Fig. 4D–E). However, Wang's study demonstrated an atherosclerotic role of TLR4 in anti-β2GPI antibody-treated ApoE$^{-/-}$mice, which seems to be opposite to our findings. The variety of results may due to the impaired lipid metabolism in ApeE$^{-/-}$ mice and the complexity of *in vivo* system that may affect the formation of β2/aβ2 [36]. Migration of macrophages from subendothelial space to lumen is defined as 'reversed-migration', which played a protective role in AS progression [37,38]. According to previous reports, FAK activation could negatively regulate the reversed-migration [39,40]. In this study, we found the reversed-migration of THP-1 macrophages was inhibited by oxLDL. Moreover, the effect of oxLDL on reversed-migration could be partly relieved by β2/aβ2 in a TLR4-dependent manner (Fig. S4). The result of macrophages reversed-migration was just in the opposite to that of FAK activation, which was in line with the result found by Park et al. [6].

OxLDL-triggered expression of PPAR-γ, a hormone nuclear receptor that set off CD36 gene transcription [41,42], was inhibited by β2/aβ2 (Fig. 5A). Moreover, CD36 localization on cell membrane is prerequisite for its endocytosis of oxLDL [43]. We found that CD36 protein mainly localized on the membrane of oxLDL-treated THP-1 macrophages, whereas localized on cytoplasm of THP-1 macrophages treated with oxLDL + β2/aβ2 (Fig. 5B). Furthermore, attenuated PPAR-γ expression and CD36 intracellular localization in THP-1 macrophages were reversed by TLR4 blockade (Fig. 5). These results explained how β2/aβ2-induced TLR4 expression negatively regulates CD36 function by

influencing both PPAR-γ level and CD36 protein localization.

Based upon these collective results, we propose that β2/aβ2 played pathological stage-related roles in AS, both of which need the involvement of TLR4. In the initial stage of AS, β2/aβ2 triggers the expression of inflammatory cytokines and MMPs in a TLR4-dependent manner, which accelerates the onset of AS by promoting vascular inflammation and ECM degradation. When AS developed into fatty streak stage, β2/aβ2 inhibited oxLDL-induced CD36 activation and subsequently the intracellular lipid accumulation, FAK activation and the reversed-migration of THP-1 macrophages in a TLR4-dependent manner, which could delay the progression of AS. In conclusion, we demonstrated the novel dual roles of β2/aβ2 and TLR4 which promotes the initiation but delays the intima lipids accumulation in AS.

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Declaration of competing interest

The Authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.117000.

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