Involvement of TLR4 in Oxidized LDL/β2GPI/Anti-β2GPI-Induced Transformation of Macrophages to Foam Cells

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Aim: It has been reported that oxidized low-density lipoprotein (oxLDL) forms a stable and non-dissociable complex with β2-glycoprotein I (β2GPI) and that IgG anti-β2GPI autoantibodies are able to recognize this complex, thus facilitating macrophage-derived foam cell formation in patients with antiphospholipid syndrome (APS). However, the immunopathological mechanisms of oxLDL/β2GPI complexes in promoting foam cell formation are not fully understood. In this study, we examined the role of toll-like receptor 4 (TLR4) in the oxLDL/β2GPI/anti-β2GPI complex-induced transformation of mouse peritoneal macrophages to foam cells.

Methods: Oil red O staining and optical density (OD) measurements of intracellular stained oil red O solution were used to monitor the transformation of peritoneal macrophages to foam cells in TLR4-competent C3H/HeN and TLR4-mutant C3H/HeJ mice. During foam cell formation induced by the oxLDL/β2GPI/anti-β2GPI complex, the expression of TLR4 and activation of nuclear factor kappa B (NF-κB) were confirmed by analyzing the protein and mRNA levels of these compounds. Furthermore, the related active molecule expression during foam cell formation induced by the oxLDL/β2GPI/anti-β2GPI complex was examined in the presence or absence of TLR4.

Results: The data showed that treatment with the oxLDL/β2GPI/anti-β2GPI complex markedly increased foam cell formation, the TLR4 expression, NF-κB activation, the tissue factor (TF) expression and tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 (MCP-1) secretion in the C3H/HeN mice. However, the transformation of macrophages to foam cells and the expression levels of phosphorylated NF-κB, TF, TNF-α and MCP-1 were significantly reduced in the C3H/HeJ mice treated with the oxLDL/β2GPI/anti-β2GPI complex. In addition, compared with that achieved by oxLDL alone, the oxLDL/β2GPI complex decreased foam cell formation and the related signaling molecule expression in the C3H/HeN mice.

Conclusions: Our results indicate that TLR4 plays an important role in the process of oxLDL/β2GPI/anti-β2GPI complex-induced transformation of macrophages to foam cells, which may accelerate the development of atherosclerosis in the setting of APS. However, β2GPI alone functions as an anti-atherogenic protein by preventing the foam cell formation induced by oxLDL.


Key words: TLR4, NF-κB, oxidized LDL/β2GPI/anti-β2GPI complex, foam cells, TF

Introduction

Atherosclerosis is a chronic inflammatory disorder and major health concern in industrialized countries. The retention and modification of lipoproteins triggers the generation of a series of oxidation prod-
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ucts, some of which promote early-stage lesion development via the recruitment of monocyte-derived macrophages in the blood vessel wall and the intracellular accumulation of cholesteryl esters in macrophages. These macrophages are termed foam cells after taking in modified lipids, in particular, oxidized LDL (oxLDL). Furthermore, as the major immune cells in lesions, macrophages promote the expression of pro-inflammatory molecules and activation of pattern recognition receptors.

Atherosclerosis is a complex, highly interconnected biologic process. Metabolic and immune mechanisms interact to initiate and promote the progression of atherosclerotic lesions. Inflammatory and immunologic responses triggered by the interaction of modified lipoproteins with pattern recognition receptors likely play important roles in the initiation of atherosclerosis, as supported by the findings of clinical and in vitro studies. As shown in a recent clinical analysis, atherosclerosis exhibits a close relationship with systemic autoimmune diseases. Moreover, accelerated lesion formation has been observed in some patients with antiphospholipid syndrome (APS) in the absence of other risk factors. It is therefore suggested that autoimmunity promotes the development of atherosclerosis.

APS is an autoimmune disease characterized by the presence of a high titer of antiphospholipid antibodies (aPL) in the plasma in affected patients. The main clinical manifestations of APS include vascular thrombosis, recurrent complications of pregnancy and arterial involvement, including coronary heart disease and peripheral vascular disease, both of which share common characteristics of atherosclerosis. The majority of aPL recognize phospholipid binding proteins directly instead of phospholipids, among which β2-glycoprotein I (β2GPI) is the major antigenic target. In 1999, oxLDL was found to co-localize with β2GPI in the cells of atherosclerotic lesions, suggesting the participation of β2GPI in atherogenesis. It was later discovered that oxLDL binds to endogenous β2GPI, thus forming a complex in the bloodstream in APS patients. Furthermore, in vitro experiments have demonstrated that oxLDL/β2GPI complexes are recognized by monoclonal anti-β2GPI antibodies and internalized by macrophages via anti-β2GPI antibody-mediated phagocytosis. The results of these studies suggest that the anti-β2GPI antibodies observed in patients with APS are likely pro-atherogenic.

The pathogenesis of APS-associated atherosclerosis acceleration is multifactorial, and various mechanisms underlying this process have been proposed. In particular, TLR4 has been studied with respect to its role in the connection between autoimmune diseases and atherosclerosis. Previous studies have demonstrated that TLR4 is expressed in both human and murine lipid-rich atherosclerotic plaques. In addition, the TLR4 present in macrophages is recognized by oxLDL and promotes monocyte-derived macrophage migration and the related inflammatory molecule expression in vitro. Importantly, a deficiency in TLR4 or its downstream adaptor molecules significantly reduces the degree of plaque accumulation and vessel inflammation, implying the importance of TLR4 and its downstream pathway in the pathology of atherosclerosis.

Although the roles of TLR4 and its downstream pathway in patients with atherosclerosis and APS have been extensively investigated, the detailed mechanisms underlying the function of these molecules in the acceleration of APS-associated atherosclerosis have not yet been explored. In this study, we assessed the effects of TLR4 and NF-κB on oxLDL/β2GPI/anti-β2GPI complex-induced foam cell formation and the vascular inflammation molecule expression in mouse peritoneal macrophages.

Materials and Methods

Animals

C3H/HeN mice (TLR4-competent), which served as controls, were purchased from Vital River Laboratory Animal Technology (Beijing, China). C3H/HeJ mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and carried a point mutation in the TLR4 gene, suggesting a defect in the expression of the functional receptor. All mouse experiments were approved by the Laboratory Animal Administration Committee of Jiangsu University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Mouse Peritoneal Macrophages

The C3H/HeN mice and C3H/HeJ mice (6 to 8 weeks of age, male) were treated with an intraperitoneal injection of 4% (w/v) sterile starch broth on days 0, 1 and 2 and killed via cervical dislocation on day 3. After being soaked in 75% alcohol for five minutes, the mice underwent lavage of the peritoneal cavity...
with 10 mL of phosphate-buffered saline (PBS) \(^{22}\). The cell suspension was centrifuged (1,000 rpm, 5 min) at 4 °C and suspended in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin and 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA). The cells were incubated in 6-well plates (2.0 × 10^6 cells/well) at 37 °C and 5% CO\(_2\) in a humidified incubator to near confluence. After being incubated for four hours, the non-adherent cells were removed, and the remaining cells were covered with warm RPMI deprived of serum for 16 hours and then used for the experiments.

### Oil Red O Staining

The macrophages obtained from the C3H/HeN and C3H/HeJ mice were incubated for 48 hours with oxLDL (20 μg/mL; Biomedical Technologies, Inc., Stourghton, MA, USA), oxLDL (20 μg/mL)/β2GPI (100 μg/mL; US Biological, Swamscott, MA, USA) complex, β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL; Chemicon, Temecula, CA, USA) complex, oxLDL (20 μg/mL)/β-GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex or LPS (500 ng/mL; Sigma, Saint Louis, Missouri, USA) followed by staining with oil red O to identify foam cells. Stock oil red O solution was made by dissolving 0.5 g oil red O powder in 100 mL of isopropanol, after which the working solution was prepared by mixing three parts stock solution with two parts deionized water followed by gravity filtration through Whatman No. 1 filter paper. The cells were fixed using 4% paraformaldehyde (PFA) and then washed with PBS and stained for 20 minutes at 37 °C with oil red O working solution and counterstained with hemotoxylin. The cell morphology was observed using an inverted optical microscope equipped with an imaging system. Foam cells were discerned by observing intracellular lipid inclusions that stained red-brown by oil red O working solution.

### Quantitative Analysis of Foam Cell Formation

The cells were seeded at a density of 5 × 10^5 into 24-well plates and serum incubated for 48 hours following stimulation with the respective stimulants mentioned above. The foam cells were stained for 20 minutes at 37 °C with oil red O working solution. The cells were washed with 60% isopropanol to remove the background color, after which the intracellular stained oil red O was eluted with isopropanol and the optical density (OD) of the solution at 520 nm was measured using a kinetic microplate reader (Gene Company Limited, Hong Kong, China) \(^{23}\). The OD values were proportional to the amount of intracellular cholesteryl esters, reflecting the extent of transformation of macrophages to foam cells.

### Western Blotting Analysis

The mouse peritoneal macrophages were treated with different stimulants similar to that described above. All reagents (except for LPS) were subjected to Detoxi-Gel™ (Pierce, Rockford, IL, USA) in order to remove endotoxin contamination (<0.03 EU/mL) using a Limulus amebocyte lysate assay (ACC, Falmouth, MA, USA). The total proteins of the cells were accumulated in accordance with the previous method. Equal amounts of proteins (5 μg) from cells treated under different experimental conditions were electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was subsequently blocked in fresh 5% dry defatted milk in Tris-buffered saline/0.05% Tween-20 (TBS/T) for two hours at room temperature, washed with TBST three times and then blotted with the primary antibodies against TLR4 (1:1,000, Biogot, Nanjing, China), NF-κB p65, phospho-p65 ser536 (1:1,000, Cell Signalling, Beverly, MA, USA) and β-actin (1:2,000, Proteintech Group, Chicago, IL, USA), respectively, overnight at 4 °C. Following three washes with TBST, the bound primary antibody was detected using species-specific horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) at a concentration of 1:2,000-1:5,000. Finally, the immunoblots were visualised using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

### Real-Time Quantitative PCR

Quantitative real-time PCR (qPCR) was used to assess the mRNA levels of TLR4 and TF following incubation of the mouse peritoneal macrophages with different stimulants similar to that described above. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Oligo dT-primers were used for reverse transcription with 2 μg of total RNA in a 25-μL reaction volume (TOYOBO Bio-Technology, Osaka, Japan; 2720 Thermal Cycler). The levels of TLR4 and TF mRNA in the cells were determined using qPCR with SYBR Green I dye (Takara Biotec, Kyoto, Japan). The primers used in PCR to obtain 123-bp TLR4 fragments were forward: 5′-AGCCCTTTCCAGGAATTAACGTC-3′ and reverse: 5′-TCCCAAGATCAACCCTATGGAC-3′; the 137-bp TF fragments were forward: 5′-TCAAGCAGGGAAGAAAAAC-3′ and reverse: 5′-CTGCTTCCCTGGGCTATTTTG-3′ and the 200-
The concentration of generated factor Xa was calculated as 405 nm using a kinetic microplate reader. The concentration was monitored according to the absorbance at activation by the TF/VIIa complex. Color development was determined based on the level of factor X. The TF activity following incubation of the cells with the respective stimulants was assessed primarily with a TF activity kit (Assaypro, Greenwich, CT, USA). Briefly, the above cell lysates were collected and assayed using the TF activity kit according to the manufacturer's instructions. The TF activity in the cells was determined based on the level of factor X activation by the TF/VIIa complex. Color development was monitored according to the absorbance at 405 nm using a kinetic microplate reader. The concentration of generated factor Xa was calculated as Vmax (mOD/minute [min]) using a standard curve.

**TF Activity Assay**

The level of TF activity following incubation of the cells with the respective stimulants was assessed based on the findings of the experimental groups. Cells were seeded at a density of 5 × 10⁵ into 24-well plates and serum starved for 16 hours prior to stimulation with the respective stimulants mentioned above. In our preliminary experiments, we confirmed that the secretion of TNF-α and MCP-1 following stimulation with three complexes reached fastigium at 12 hours and then entered a platform based on an analysis of the cell culture supernatants. Therefore, we collected the cell supernatants in the treatment groups at 12 hours in order to determine the levels of TNF-α and MCP-1 using enzyme-linked immunosorbent (ELISA) assay kits (BOSTER, WuHan, China) according to the manufacturer’s instructions.

**Statistical Analysis**

All experimental points were performed in triplicate or quadruplicate, and all assays were repeated a minimum of three times. Normally distributed variables were expressed as the mean ± standard deviation (SD). Differences between the control and experimental conditions were assessed using the Student’s two-tailed t-test for paired samples. For multiple group comparisons, we used ANOVA with Dunnett’s post test. All statistical analyses were performed using the SPSS statistical software package, version 17.0. Statistical significance was defined as p < 0.05.

**Results**

**TLR4 Promotes oxLDL/β2GPI/Anti-β2GPI Complex-Induced Macrophage-Derived Foam Cell Formation**

Oil red O solution was used to stain intracytoplasmic lipid droplets to confirm the macrophage uptake of oxLDL. Macrophages obtained from TLR4-competent C3H/HeN and TLR4-mutant C3H/HeJ mice were incubated with oxLDL, oxLDL/β-GPI complex, β-GPI/anti-β-GPI complex, oxLDL/β-GPI/anti-β-GPI complex or LPS for 48 hours before staining. As shown in Fig. 1, the oxLDL/β-GPI/anti-β-GPI complex enhanced the uptake of lipids in the macrophages obtained from the C3H/HeN mice, as demonstrated by a more intense red color and higher OD values than that observed in the cells treated with oxLDL or the oxLDL/β-GPI complex. It appeared that the oxLDL/β-GPI complex even reduced the macrophage uptake of lipids, producing a less intense red color and decreased OD values compared to oxLDL alone (Fig. 1A, B). In addition, more foam cell formation was observed and the OD values were elevated following incubation with oxLDL, the oxLDL/β-GPI complex or the oxLDL/β-GPI/anti-β-GPI complex in the C3H/HeN macrophages compared to that observed in the C3H/HeJ macrophages (Fig. 1A, B). However, treatment without oxLDL did not induce foam cell formation in either mouse strain (Fig. 1A, B).

**The oxLDL/β-GPI/Anti-β-GPI Complex Increases the TLR4 Expression in C3H/HeN Macrophages**

In order to further investigate the role of TLR4 in oxLDL/β-GPI/anti-β-GPI complex-induced C3H/HeN foam cell formation, we analyzed the TLR4 expression at both the protein and mRNA levels. The cells were treated with oxLDL, the oxLDL/β-GPI complex, the β-GPI/anti-β-GPI complex, the oxLDL/β-GPI/anti-β-GPI complex or LPS, as described in the Materials and methods section. After two hours of treatment, the cells were collected for total RNA extraction and real-time quantitative PCR. After six hours, the cell lysates were prepared for a Western blotting analysis. The results showed that treatment with oxLDL, the β-GPI/anti-β-GPI complex, the oxLDL/β-GPI/anti-β-GPI complex or LPS significantly increased the expression of TLR4. The stimulatory effect of the oxLDL/β-GPI/anti-β-GPI complex was strongest (p < 0.05 vs control). Interestingly, the TLR4 expression induced by the oxLDL/β-GPI complex was less significant than that induced by oxLDL alone (p < 0.05) (Fig. 2A, B).
The OxLDL/β2GPI/anti-β2GPI complex induced the conversion of macrophages into foam cells and the involvement of TLR4. Peritoneal macrophages (2.0 × 10⁶) obtained from C3H/HeN mice and C3H/HeJ mice were treated with oxLDL (20 μg/mL), oxLDL (20 μg/mL)/β2GPI (100 μg/mL), β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex, oxLDL (20 μg/mL)/β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex and LPS (500 ng/mL) for 48 hours. Foam cells were identified with oil red O staining for intracellular lipids (A) and quantitated with optical density (OD) measurements of intracellular stained oil red O solution (B). The cells were visualized using light microscopy (×200). The OD values of the solution at 520 nm were measured using a kinetic microplate reader. Shown are the representative results of three separate experiments with similar results. *p < 0.05 vs control of untreated cells (0 h or media), **p < 0.05 cells treated with oxLDL/β2GPI complex vs cells treated with oxLDL in C3H/HeN mice, #p < 0.05 vs same stimulation group in C3H/HeN mice.
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its downstream signaling molecule NF-κB are involved in the β2GPI/anti-β2GPI-induced TF expression in monocytes. In order to further investigate whether its phosphorylation is partially dependent on TLR4 during foam cell formation in C3H/HeN mice.

The Phosphorylation of NF-κB is Partially Dependent on TLR4 During Foam Cell Formation

Our previous study demonstrated that TLR4 and

Fig. 2. The OxLDL/β2GPI/anti-β2GPI complex upregulated the TLR4 expression during the process of macrophage differentiation to foam cells in the C3H/HeN mice. Macrophages (2.0 × 10^6) were treated with OxLDL (20 μg/mL), OxLDL (20 μg/mL)/β2GPI (100 μg/mL), β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex, OxLDL (20 μg/mL)/β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex, and LPS (500 ng/mL) for six or one hour, respectively. The cell lysates (at 6 h) and total RNA of the cells (at 1 h) were collected. The protein levels of TLR4 (A) were analyzed using a Western blotting analysis, and the mRNA levels of TLR4 (B) were detected using quantitative real-time PCR. Shown are the pooled data of three separate experiments with similar results. *p < 0.05 vs control of untreated cells (media).

Fig. 3. The involvement of TLR4 in the OxLDL/β2GPI/anti-β2GPI complex activated NF-κB p65 during foam cell formation.

(A) Macrophages (2.0 × 10^6) were treated with OxLDL (20 μg/mL)/β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex for different times, as indicated. (B) The cells were stimulated with OxLDL (20 μg/mL), OxLDL (20 μg/mL)/β2GPI (100 μg/mL), β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex, OxLDL (20 μg/mL)/β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex and LPS (500 ng/mL) for one hour. The cell lysates were collected in order to analyze the phosphorylation of NF-κB p65 using Western blotting. Shown are the representative results of three separate experiments with similar results. *p < 0.05 vs control of untreated cells (media).

* p < 0.05 vs control of untreated cells (0 h or media). ** p < 0.05 cells treated with OxLDL/β2GPI complex vs cells treated with OxLDL in C3H/HeN mice. *p < 0.05 vs same stimulation group in C3H/HeN mice.
NF-κB is also activated during the foam cell formation induced by the oxLDL/β2GPI/anti-β2GPI complex, the level of NF-κB p65 phosphorylation was examined. As shown in Fig. 3A, the oxLDL/β2GPI/anti-β2GPI complex induced the phosphorylation of NF-κB p65 in the C3H/HeN mouse macrophages in a time-dependent manner. The phosphorylation of NF-κB p65 gradually increased following stimulation, reaching a maximal level at 60 minutes then gradually decreasing (Fig. 3A).

Next, we explored the interaction between NF-κB and TLR4 during the process of oxLDL/β2GPI/anti-β2GPI complex-induced transformation of macrophages to foam cells. In the C3H/HeN mice, NF-κB was activated in macrophages incubated with oxLDL, the β2GPI/anti-β2GPI complex, the oxLDL/β2GPI/anti-β2GPI complex or LPS (p < 0.05 vs control). The effect of the oxLDL/β2GPI/anti-β2GPI complex was more prominent than that of oxLDL or the β2GPI/anti-β2GPI complex. Compared with that observed in the C3H/HeN macrophages, the levels of activation of NF-κB in the C3H/HeJ macrophages following the same treatments were significantly lower (p < 0.05) (Fig. 3B). Furthermore, the oxLDL/β2GPI complex barely promoted NF-κB activation in the macrophages obtained from both mouse strains (p > 0.05 vs control).

The TF Expression in the C3H/HeN and C3H/HeJ Mice During Foam Cell Formation

In order to study the TF expression during oxLDL/β2GPI/anti-β2GPI complex-induced foam cell formation, we analyzed the TF mRNA and activity in macrophages obtained from the C3H/HeN and C3H/HeJ mice after stimulation. Total RNA was extracted from the cells after two hours of treatment, and the cell lysates were collected after six hours of treatment for the TF activity assay. The results showed that the TF mRNA expression and TF activity induced by the oxLDL/β2GPI/anti-β2GPI complex were both increased in the C3H/HeN mice (p < 0.05 vs control), similar to that observed in the LPS-treated group. However, the oxLDL/β2GPI complex had little effect on the TF expression, whose level was much lower than that observed following treatment with oxLDL alone (p < 0.05 vs oxLDL). In contrast, in the C3H/HeJ mice, no treatments had a significant effect on either the TF mRNA expression or TF activity (p > 0.05 vs control) (Fig. 4A, B).
Effects of TLR4 on the Expression of TNF-α and MCP-1 in the Mouse Macrophages Stimulated by the oxLDL/β2GPI/Anti-β2GPI Complex

We further investigated whether mouse macrophage-specific dysfunction of TLR4 has any impact on inflammation or chemotaxis induced by the oxLDL/β2GPI/anti-β2GPI complex. The results showed that the TLR4 mutation in macrophages evidently reduced the TNF-α and MCP-1 levels in the cell supernatants (p<0.05) (Fig. 5A, B). The data also demonstrated that the oxLDL/β2GPI/anti-β2GPI complex produced the greatest effect on the TNF-α and MCP-1 levels and that oxLDL exerted a stronger stimulatory effect than the oxLDL/β2GPI complex (p<0.05 vs oxLDL) (Fig. 5A, B).

Discussion

Multiple processes have been implicated in the pathophysiology of atherosclerosis, including lipid metabolism, inflammation, coagulation activation and immune reactions. Many clinical studies have demonstrated that these factors are interdependent. The association between autoimmunity and atherosclerosis is widely accepted, and recent studies have shown that antiphospholipid antibody positivity is a risk factor for subsequent subclinical atherosclerosis and may play an important role in the pathogenesis of atherosclerosis25, 26). Moreover, Kobayashi et al. and Kajiwara et al. reported that β2GPI binds with Cu²⁺-oxLDL via its ligands ω-carboxylated-7-ketocholesteryl esters and that oxLDL/β2GPI complexes are stable and accumulate at high serum levels in APS patients with arterial thrombosis27). In recent studies by these authors, it was found that anti-β2GPI associates with oxLDL/β2GPI complexes before being internalized by macrophages mediated by Fcγ receptors and scavenger receptors10, 11, 28, 29). Although Fcγ receptors and scavenger receptors have been confirmed to be involved in the process of macrophage internalization of the oxLDL/β2GPI/anti-β2GPI complex, the roles of TLR4 and its downstream signaling molecules are not fully understood.

Our previous studies demonstrated that TLR4 acts as a cofactor for annexin A2 on the surface of monocytes and contributes to the β2GPI/anti-β2GPI complex-enhanced TF expression30, 31). In the current study, we investigated whether TLR4 participates in oxLDL/β2GPI/anti-β2GPI complex-induced foam cell formation. The present results demonstrated that the oxLDL/β2GPI/anti-β2GPI complex promotes the formation of macrophage-derived foam cells and enhances the TLR4 expression in C3H/HeN mice (Fig. 1, 2). It was also observed that the oxLDL/β2GPI/anti-β2GPI complex-induced TNF-α and MCP-1 secretion during the formation of foam cells.

Macrophages (5.0 × 10⁵) were treated with oxLDL (20 μg/mL), oxLDL (20 μg/mL)/β2GPI (100 μg/mL), β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex, oxLDL (20 μg/mL)/β2GPI (100 μg/mL)/anti-β2GPI (10 μg/mL) complex and LPS (500 ng/mL) for 12 hours. The cell culture supernatants were collected in order to analyze the secretion of TNF-α (A) and MCP-1 (B) using commercial ELISA kits. Shown are the representative results of three separate experiments with similar results. *p<0.05 vs control of untreated cells (0 h or media), **p<0.05 cells treated with oxLDL/β2GPI complex vs cells treated with oxLDL in C3H/HeN mice, #p<0.05 vs same stimulation group in C3H/HeN mice.

Fig. 5. TLR4 in the oxLDL/β2GPI/anti-β2GPI complex induced TNF-α and MCP-1 secretion during the formation of foam cells.
β2GPI/anti-β2GPI complex induced a small amount of foam cell formation in the TLR4-mutant C3H/HeJ mice. These results indicate that the oxLDL/β2GPI/anti-β2GPI complex promotes foam cell transformation, a process that partly depends on TLR4. The inhibition of TLR4 may therefore be beneficial with respect to the clinical control of accelerated atherosclerosis in patients with APS.

It has also been demonstrated that NF-κB plays an important role in the control of cell proliferation, survival and invasion as well as angiogenesis. We previously demonstrated that the activation of NF-κB p65 is a process mediated by multiple receptors, although the activation of NF-κB p65 by LPS and anti-β2GPI/β2GPI is primarily mediated by TLR4.

In the current study, we further confirmed that NF-κB is activated during the foam cell formation induced by the oxLDL/β2GPI/anti-β2GPI complex (Fig. 3). Therefore, the TLR4/NF-κB signaling pathway that is active during oxLDL/β2GPI/anti-β2GPI complex-induced foam cell formation may play a role in the pathological development of atherosclerosis in APS patients. However, the activation of NF-κB is not entirely dependent on TLR4, suggesting that other signaling pathways may be involved in this process.

Recently, it has been shown that early vascular alterations and endothelial cell activation biomarkers are more pronounced in patients with SLE, which is in line with the high cardiovascular risks observed in such patients. An elevated level of whole blood TF procoagulant correlates with the increased formation of β2GPI/anti-β2GPI complexes in monocytes. However, the changes in TF and related signaling pathways in APS patients with accelerated atherosclerosis are largely unknown. In this study, we found that both the TF expression and activity were increased during foam cell formation induced by the oxLDL/β2GPI/anti-β2GPI complex in C3H/HeN mice. However, these changes were not observed in the C3H/HeJ mice. These data strongly suggest that the TF expression in macrophages induced by the oxLDL/β2GPI/anti-β2GPI complex is largely dependent on the activation of TLR4. Based on our results, it is reasonable to conclude that TLR4 and anti-β2GPI antibodies play important roles in the acceleration of APS-associated atherosclerosis by promoting the TF expression and activation.

While macrophages can turn into large foam cells after migrating to the subendothelial intima and internalizing oxLDL, they also produce cytokines, such as tumor necrosis factor-α (TNF-α), and chemokines, such as monocyte chemotactic protein-1 (MCP-1). As shown in recent studies, both cytokines and chemokines promote the formation and development of atherosclerotic plaque as well as intravascular thrombi in patients with autoimmune diseases. Although the involvement of cytokines and chemokines in the pathogenesis of atherosclerosis and autoimmune diseases is widely accepted, little is known about the underlying mechanisms or how these molecules trigger vascular inflammation and interact with autoantibodies. Our data indicate that TLR4 gene mutations result in reduced TNF-α and MCP-1 levels in the cell culture supernatants. Therefore, the oxLDL/β2GPI/anti-β2GPI complex may contribute to cytokine and chemokine production via the actions of TLR4. However, we cannot exclude the possibility that the oxLDL/β2GPI/anti-β2GPI complex also promotes the activation of macrophages through other receptors, such as TLR3, a scavenger receptor. It has previously been shown that TLR3 elicits different sets of inflammatory mediators as a pro-atherogenic receptor in hematopoietic immune cells and is a potential therapeutic target in patients with atherosclerotic cardiovascular disease. Whether TLR3 and scavenger receptors are involved in this process should be investigated in future studies.

Hasunuma et al. found that β2GPI inhibits in vitro uptake, cell surface binding, cellular associations and the proteolytic degradation of oxLDL in mouse macrophages, indicating that β2GPI is an anti-atherogenic factor. Interestingly, we demonstrated that oxLDL/β2GPI inhibits the activation of TLR4, its uptake by macrophages, the activation of NF-κB and the expression of TF, TNF-α and MCP-1 in TLR4-competent C3H/HeN mice. However, many studies have also indicated that oxLDL/β2GPI complexes are associated with the severity of CAD and increase the rate of development of autoimmune-mediated atherosclerosis lupus. Combining these findings with our results, it is strongly suggested that there are double roles for β2GPI in foam cell formation. Furthermore, it appears that β2GPI exert an anti-atherosclerotic activity by preventing oxLDL-induced foam cell formation in the absence of antiphospholipid antibodies, which may explain why the β2GPI present in healthy human plasma does not increase the risk of atherosclerosis. On the other hand, β2GPI increases oxLDL uptake and the expression of relevant active molecules, which subsequently exerts an atherogenic effect in the presence of antiphospholipid antibodies.

In conclusion, our data demonstrate that TLR4 not only plays an important role in the formation of foam cells induced by the oxLDL/β2GPI/anti-β2GPI complex, but also contributes to vascular thrombosis and inflammation in mice. In the present study, the
activation of the TLR4 downstream signaling molecule NF-κB was not completely inhibited by TLR4 gene mutation. Future studies are needed to elucidate the exact mechanisms by which the TLR4-dependent activation of downstream signaling molecules contributes to the pathophysiology of atherosclerosis. In addition, based on our previous study, whether TLR4 acts as a cofactor for annexin A2 on macrophages and contributes to oxLDL/β2GPI complex-induced foam cell formation should be further investigated\(^{30, 31}\). Moreover, the contribution of TLR4 and anti-β2GPI antibodies to the pathophysiology of accelerated APS-related atherosclerosis should be revisited and investigated in clinical studies using TLR4 antagonist agents. Such future studies should focus on treatment as well as prevention, early diagnosis, personalized medication and prognosis.

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**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

**List of Abbreviations**

TLR4: toll-like receptor 4  
oxLDL: oxidized low-density lipoprotein  
β2GPI/anti-β2GPI: β2-glycoproteinI/anti-β2-glycoprotein I  
APS: antiphospholipid syndrome  
NF-κB: nuclear factor kappa B  
aPL: antiphospholipid antibodies  
TF: tissue factor  
TNF-α: tumor necrosis factor-α  
MCP-1: monocyte chemoattractant protein-1  
SLE: systemic lupus erythematosus  
CAD: coronary artery disease

**References**

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