Epigallocatechin-3-gallate inhibits TF and TNF-α expression induced by the anti-β_2GPI/β_2GPI complex in human THP-1 cells

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Abstract. Epigallocatechin-3-gallate (EGCG) is the major polyphenolic component of green tea. The aim of the current study was to investigate the inhibitory effects of EGCG on anti-β_2-glycoprotein I (β_2GPI)/β_2GPI-induced tissue factor (TF) and tumor necrosis factor-α (TNF-α) expression in the human acute monocytic leukemia cell line, THP-1, as well as the underlying mechanisms. Human THP-1 cells cultured *in vitro* were treated with lipopolysaccharide (LPS, 500 ng/ml) or with the anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex following pre-treatment with or without EGCG (0-50 μg/ml). The expression levels of TF, TNF-α and Toll-like receptor 4 (TLR4) were measured, and the activation of mitogen-activated protein kinases (MAPKs) including p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), and the nuclear factor-κB (NF-κB) signaling pathway was determined by western blot analysis. The results revealed that the anti-β_2GPI/β_2GPI complex activated the THP-1 cells, resulting in the enhanced expression of the coagulation cytokine, TF, as well as that of the pro-inflammatory cytokine, TNF-α; these levels were almost comparable to those induced by LPS. Pre-treatment with EGCG decreased the TF and TNF-α levels in the THP-1 cells treated with the anti-β_2GPI/β_2GPI complex in a dose-dependent manner and counteracted the upregulation of TLR4 expression (mRNA and protein) which was induced by the anti-β_2GPI/β_2GPI complex or LPS. Furthermore, EGCG suppressed the phosphorylation of p38, ERK1/2 and JNK and blocked the activation of the NF-κB signaling pathway induced by the anti-β_2GPI/β_2GPI complex or LPS. In conclusion, our results indicate that EGCG decreases the anti-β_2GPI/β_2GPI-induced TF and TNF-α expression in THP-1 cells possibly through the inhibition of the intracellular signal transduction pathway of TLRs-MAPKs-NF-κB axis and may serve as a preventive and therapeutic agent for antiphospholipid syndrome (APS).

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder caused by the production of antiphospholipid antibodies (aPLs) which contribute to thrombosis (1). In addition to anionic phospholipids, aPLs also recognize phospholipid binding proteins, including β_2-glycoprotein I (β_2GPI) and prothrombin (2). Among these, β_2GPI has emerged as the major antigenic target for aPLs. Anti-β_2GPI antibodies are found abundantly in the plasma of patients with APS, suggesting its important role in the pathophysiology of APS (3).

The anti-β_2GPI/β_2GPI complex activates endothelial cells and monocytes upon binding to the surface membrane of endothelial cells and monocytes, promoting tissue factor (TF) activity, thereby increasing the risk of thrombosis, and enhancing the expression and secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, which are beneficial to thrombus formation in APS (4-6). The stimulation of endothelial cells or monocytes by the anti-β_2GPI/β_2GPI complex has been shown to be mediated by intracellular pathways dependent on certain receptors, such as Toll-like receptor 4 (TLR4), which help ligand recognition and binding. As a pathogen recognition protein, the activation of TLR4 by its natural ligand, lipopolysaccharide (LPS), or by other ligands, plays an important role in activating the innate immune system (7), in recognizing microbes and in initiating inflammatory responses. In recent studies, we revealed that TLR4 and its signal transduction pathway contribute to anti-β_2GPI/β_2GPI complex-induced TF and TNF-α expression in THP-1 cells and monocytes (8), and that the anti-β_2GPI/β_2GPI complex upregulates TF expression in THP-1 cells or monocytes following the activation of nuclear transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (9). As crucial downstream molecules of the TLR4 signaling pathway, mitogen-activated protein kinases (MAPKs), including p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) are involved in the development of a number of diseases (10), and are activated by the anti-β_2GPI/β_2GPI complex through myeloid differentiation protein 2 (MD-2) and

myeloid differentiation factor 88 (MyD88) in THP-1 cells (11). These results led us to hypothesize that the intracellular signal transduction pathway of TLRs-MAPKs-NF-κB in the anti-β_2GPI/β_2GPI complex-induced activation of cells may be a potential therapeutic target for APS.

Polyphenols of green tea, which comprise 30% of the dry weight of green tea leaves, include epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC). Among these, EGCG is the most abundant catechin and has a variety of biological and pharmacological properties, preventing cancer, allergies, oxidation, microbes, thrombosis, inflammation and cardiovascular diseases. Previous studies have demonstrated that EGCG exerts a number of beneficial effects by affecting a wide array of signal transduction pathways, including Notch (12), Wnt (13), JAK/STAT (14) and MAPK (15).

It is known that EGCG has beneficial effects; however, whether it affects the anti-β2GPI/β2GPI complex-stimulated activation of THP-1 cells remains to be determined. In the present study, we investigated the ability of EGCG to block the effects of the anti-β_2GPI/β_2GPI complex on THP-1 cells and the possible mechanisms involved in this process.

Materials and methods

Cell lines and cell culture. The human acute monocytic leukemia cell line, THP-1, was obtained from Shanghai Institutes Biological Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 1% glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco-BRL). The cells were cultured at 37˚C in a humidified incubator supplemented with 5% CO_2 near confluence and deprived of serum for 16 h prior to being used in the experiments. All experimental data were obtained from cells at passages 3-10.

Quantitative reverse transcription PCR (qRT-PCR). THP-1 cells were seeded at $2x10^6$ cells/well into 6-well plates and serum-starved for 16 h prior to stimulation with monoclonal anti-β_2GPI (10 μg/ml; Chemicon, Temecula, CA, USA)/β_2GPI (100 μg/ml; US Biological, Swampscott, MC, USA) complex, anti-β_2GPI (10 μg/ml)/bovine serum albumin (BSA) (100 μg/ml; Sigma, St. Louis, MO, USA), control rabbit immunoglobulin G isotype (R-IgG) (10 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)/β_2GPI (100 μg/ml) or 500 ng/ml of LPS (*Escherichia coli*, strain 0128:B12; Sigma) for 2 h [the concentrations of the above reagents are based on those described in our previous studies (16-18)]. Cells in some wells were pre-treated with various concentrations of EGCG (0-50 μg/ml; Sigma) for 1 h, and EGCG was not removed. Subsequently, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Oligo(dT)-primers were used for reverse transcription with 2 μg of total RNA in a 25 μl reaction volume using a 2720 Thermal Cycler (Toyobo Biotechnology, Osaka, Japan). The expression levels of the target mRNA in the cells were analyzed by qRT-PCR using SYBR-Green I dye (Takara Bio, Kyoto, Japan). The primers used for PCR were as follows: TF forward, 5'-TCAGGTG ATCCACCCACCTT-3' and reverse, 5'-GCACCCAATTT CCTTCCATTT-3'; TNF-α forward, 5'-CCCAGGCAGTCA GATCATCTTCT-3' and reverse, 5'-ATGAGGTACAGGCCC TCTGAT-3'; TLR4 forward, 5'-CCTGTGCAATTTGACC ATTG-3' and reverse, 5'-AAGCATTCCCACCTTTGTTG-3'. Primers for the control housekeeping gene β-actin were forward, 5'-CACGAAACTACCTTCAACTCC-3' and reverse, 5'-CATACTCCTGCTTGCTGATC-3'. Each pair of primers was shown to yield only one product. The amplifications were performed in triplicate on a Mx3000P qPCR System (Agilent Technologies, Santa Rosa, CA, USA) for 35 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 60˚C for TF and TLR4, 58˚C for TNF-α and 56˚C for β-actin, and extension for 30 sec at 72˚C. The relative mRNA levels of target genes to the control β-actin were calculated using a standard curve.

Detection of TNF-α secretion. THP-1 cells were seeded at $2x10^6$ cells/well into 6-well plates and serum-starved for 16 h prior to stimulation with anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex, anti-β_2GPI (10 μg/ml)/ BSA (100 μg/ml), R-IgG (10 μg/ml)/β_2GPI (100 μg/ml) or 500 ng/ml of LPS for 24 h. The cells in some wells were pre-treated with various concentrations of EGCG (0-50 μg/ml) for 1 h, and EGCG was not removed. TNF-α protein, secreted into the cell culture medium, was measured using the TNF-α ELISA kit (Neobioscience, Shenzhen, China), following the manufacturer's instructions. The TNF-α protein concentration in the cell culture medium was expressed as pg/ml.

Measurement of TF activity. THP-1 cells $2x10^6$ cells/well were treated as described above for the indicated periods of time. Cell lysates were collected and assayed using TF activity kits (Assaypro, Greenwich, CT, USA) according to the manufacturer's instructions. The TF activity in the cells was determined as factor X activation by the TF/VIIa complex as described in our previous studies (16-18). The color development in the assay was monitored by the absorbance at 405 nm using a kinetic microplate reader (Gene Co., Ltd., Hong Kong, China). The concentration of generated factor Xa was calculated as Vmax (mOD/min) using a standard curve.

Western blot analysis. The THP-1 cells were seeded at $2x10^6$ cells/well into 6-well plates and serum-starved for 16 h prior to stimulation with the complex of monoclonal anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml), LPS (500 ng/ml) for 6 h. The cells in some wells were pre-treated with various concentrations of EGCG (0-50 μg/ml) for 1 h, and EGCG was not removed. For the determination of total cellular protein, the cells were collected and lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 2.5 mM EDTA and 1 mM PMSF. The lysates were centrifuged at 10,000 rpm for 30 min using the Compact High Speed Refrigerated Centrifuge 6930. (Kubota, Tokyo, Japan) to remove unbroken cells, nuclei and other organelles. The supernatant containing plasma membrane was recovered and stored at -70˚C for analysis. Equal amounts of protein (5 μg) were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in fresh 5% dry non-fat milk in

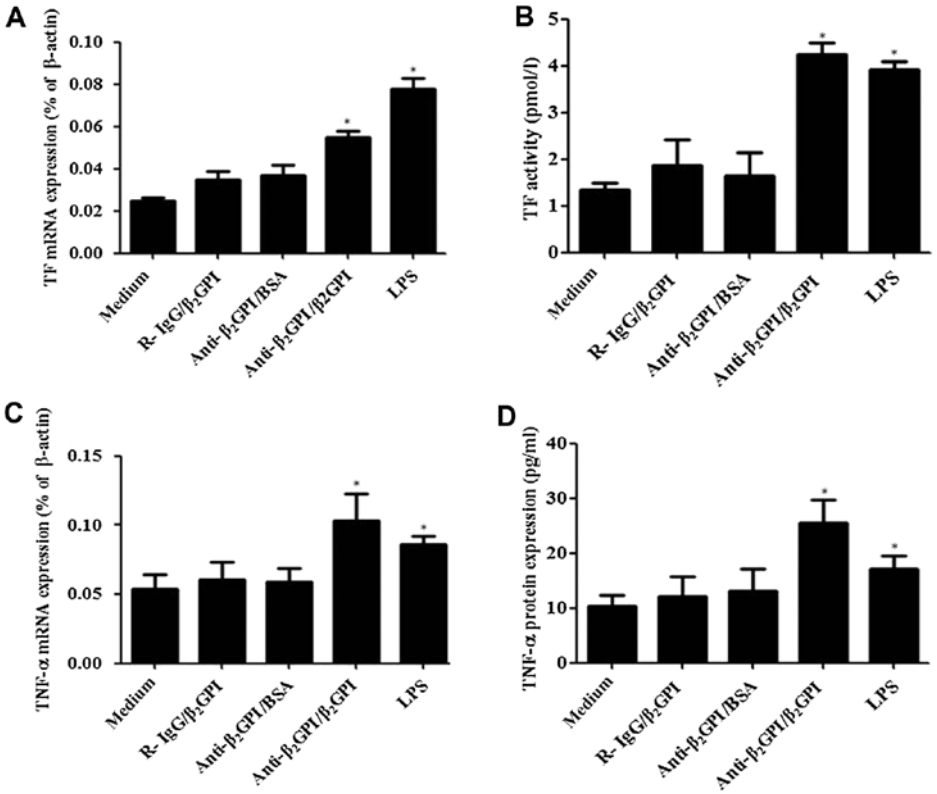

Figure 1. Anti-β_2-glycoprotein I (β_2GPI)/β_2GPI complex induces the expression of tissue factor (TF) and tumor necrosis factor-α (TNF-α) in THP-1 cells THP-1 cells ($2x10^6$) were treated with anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex, rabbit immunoglobulin G (R-IgG; 10 μg/ml)/β_2GPI (100 μg/ml), anti-β_2GPI (10 μg/ml)/bovine serum albumin (BSA) (100 μg/ml) and lipopolysaccharide (LPS) (500 ng/ml) for the indicated periods of time. Total RNA of cells (at 2 h) or cell lysates (at 6 h) or cultural supernatants (at 24 h) was collected. (A) TF mRNA and (C) TNF-α mRNA levels were detected by qRT-PCR. (B) TF activity and (D) TNF-α protein levels were analyzed using the commercial kits as described in Materials and methods. Data are presented as the means ± SEM from 3 independent experiments. *P<0.05 vs. control (untreated cells, medium only; medium).

Tris-buffered saline/0.05% Tween-20 (TBST) for 1 h at room temperature, washed with TBST 3 times, and then incubated with the primary antibodies against p38 MAPK, phospho-p38 MAPK (p-p38), ERK1/2, phospho-ERK1/2 (p-ERK1/2), JNK, phospho-JNK (p-JNK), NF-κB (p65), phospho-NF-κB (p-p65), IκB-α (1:1,000; Cell Signaling Technology, Beverly, MA, USA), TLR4 (1:500; eBioscience, San Diego, CA, USA) and β-actin (1:2,500; Proteintech Group, Inc., Chicago, IL, USA) overnight at 4˚C. Following 3 washes with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Finally, the immunoblot signals were developed using ECL detection reagents (Millipore, Billerica, MA, USA), imaged and quantified using a Bio-Rad Fluor-S MultiImager (Typhoon 9400; Amersham, Uppsala, Sweden).

Statistical analysis. Data are expressed as the means ± SEM. The statistically significant differences were calculated by applying analysis of variance (ANOVA) using SPSS software (version 16.0). Values of P<0.05 were considered to indicate statistically significant differences.

Results

Anti-β_2GPI/β_2GPI complex induces TF and TNF-α expression in THP-1 cells. Treatment of the THP-1 cells with the anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex significantly enhanced the TF mRNA levels (Fig. 1A), and its activity (Fig. 1B) compared to the untreated cells (P<0.05). TNF-α mRNA (Fig. 1C) and protein levels (Fig. 1D) were also increased (P<0.05 vs. medium only), and these effects were comparable to those induced by LPS (500 ng/ml). However, TF and TNF-α expression did not increase in the cells treated with anti-β_2GPI (10 μg/ml)/BSA (100 μg/ml) or R-IgG/β_2GPI at the same concentration as anti-β_2GPI/β_2GPI. These results were similar to those obtained in our previous studies using THP-1 cells (16-18).

EGCG inhibits TF expression induced by anti-β_2GPI/β_2GPI complex in THP-1 cells. In this study, we first investigated whether EGCG decreases the effects of anti-β_2GPI/β_2GPI complex-induced TF expression in THP-1 cells. The cells were treated with various concentrations of EGCG (0-50 μg/ml) and then stimulated with anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex for the indicated periods of time. Pre-treatment with EGCG (0-50 μg/ml) inhibited anti-β_2GPI/β_2GPI complex-induced TF expression and activation in a dose-dependent manner, showing statistical significance at 20-50 μg/ml [P<0.05 vs. control (medium only)] (Fig. 2A and B). The maximal inhibition rate of EGCG (50 μg/ml) on TF mRNA expression and activity was approximately 48 and 50%, respectively.

We then explored the specific effects of EGCG on anti-β_2GPI/β_2GPI complex-enhanced TF expression in THP-1 cells.

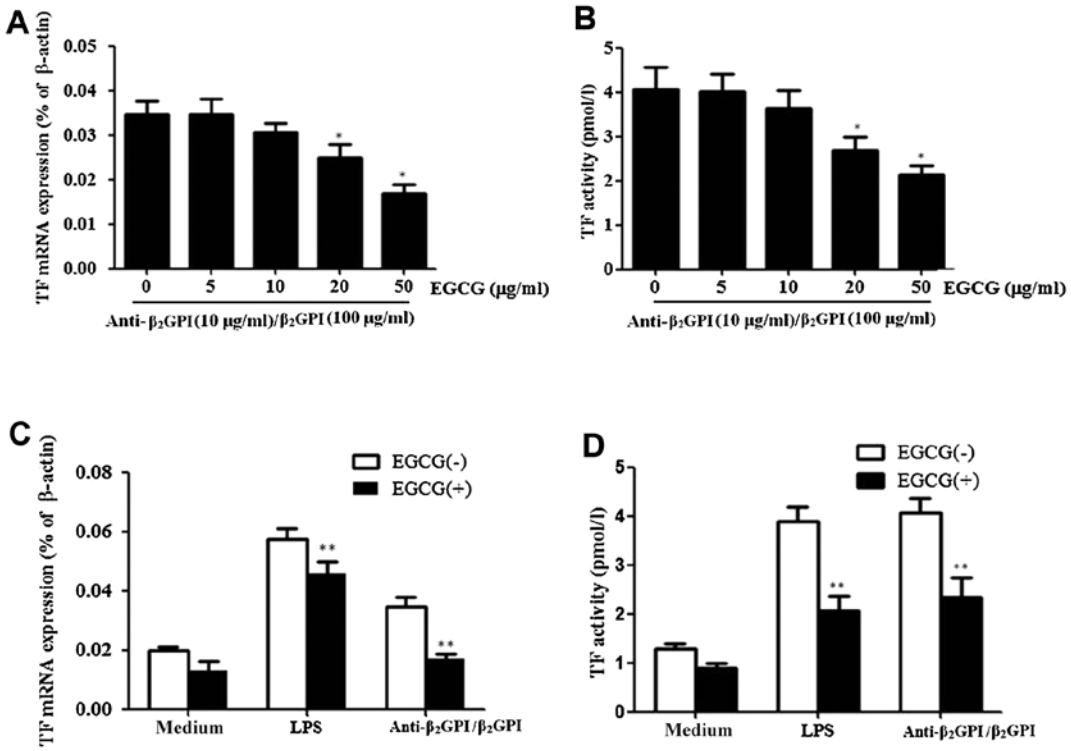

Figure 2. Effects of epigallocatechin-3-gallate (EGCG) on the mRNA expression and activity of tissue factor (TF) in THP-1 cells. Cells ($2x10^6$) were pre-treated with or without the indicated concentrations of EGCG for 1 h and then incubated with anti-β_2-glycoprotein I (β_2GPI) (10 μg/ml)/β_2GPI (100 μg/ml) complex for 2 or 6 h, as indicated. On the other hand, other THP-1 cells ($2x10^6$) were incubated with anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex or lipopolysaccharide (LPS) (500 ng/ml) in the absence or presence of EGCG (50 μg/l) for the indicated periods of time. EGCG was not removed from the medium. Total RNA of cells (2 h) or cell lysates (6 h) was collected. (A and C) TF mRNA expression and (B and D) activity were detected by qRT-PCR using commercial kits as described in Materials and methods. Data are presented as the means ± SEM from 3 independent experiments. *P<0.05 vs. cells treated with medium only (medium); **P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone.

Pre-treatment with 50 μg/ml EGCG significantly reduced the anti-β_2GPI/β_2GPI complex- or LPS-enhanced TF mRNA levels in THP-1 cells (P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone) (Fig. 2C). In addition, the enhanced TF activity level by the anti-β_2GPI/β_2GPI complex or LPS was also reduced by the presence of EGCG (50 μg/ml) (Fig.2D) (P>0.05). EGCG alone had no significant inhibitory effect on the TF level.

EGCG inhibits TNF-α expression induced by the anti-β_2GPI/β_2GPI complex in THP-1 cells. In order to investigate the effects of EGCG on the expression of TNF-α induced by the anti-β_2GPI/β_2GPI complex, the cells were pre-treated with EGCG (0-50 μg/ml) prior to stimulation with the anti-β_2GPI/β_2GPI complex for the indicated periods of time. Pre-treatment with EGCG (5-50 μg/ml) inhibited the mRNA expression levels of TNF-α in response to the anti-β_2GPI/β_2GPI complex in a dose-dependent manner, presenting statistical significance at 20-50 μg/ml (P<0.05 vs. control) (Fig. 3A). EGCG also inhibited the protein expression of TNF-α induced by the anti-β_2GPI/β_2GPI complex (Fig. 3B). The maximal inhibition rate of TNF-α mRNA and protein by EGCG (50 μg/ml) was approximately 51 and 30%, respectively.

We further determined the specific effects of EGCG on TNF-α expression in THP-1 cells. Pre-treatment with 50 μg/ml EGCG significantly reduced the anti-β_2GPI/β_2GPI complex-enhanced TNF-α mRNA and protein expression levels (Fig. 3C and D) (P<0.05 vs. anti-β_2GPI/β_2GPI complex stimulation alone). Similarly, pre-treatment with 50 μg/ml EGCG significantly decreased the LPS induced TNF-α mRNA and protein expression levels (P>0.05). By contrast, EGCG alone had no significant effects on the TNF-α level (medium only).

Effect of EGCG on the expression of TLR4 in THP-1 cells. TLR4, a family of integral membrane proteins, has been reported to mediate aPL-induced endothelial cell or monocytic cell activation (19). We have previously demonstrated that TLR4 and its signal transduction pathway contribute to anti-β_2GPI/β_2GPI complex-induced TF and TNF-α expression in THP-1 cells (8). In this study, to examine whether EGCG blocks the effects of the anti-β_2GPI/β_2GPI complex on TLR4 expression in THP-1 cells, TLR4 mRNA and protein levels in these cells were evaluated under different conditions. We found that both the anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex and LPS (500 ng/ml) increased the mRNA levels of TLR4 (Fig. 4A, white column) (P<0.05 vs. medium only). However, pre-incubation of the THP-1 cells with EGCG (50 μg/ml) significantly decreased TLR4 mRNA expression (P<0.05), even though the cells were treated with similar concentrations of the anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex or LPS (Fig. 4A, black column). Similarly, the TLR4 protein expression levels decreased following treatment with EGCG prior to stimulation with the anti-β_2GPI/β_2GPI complex or LPS (Fig. 4B) (P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone). Compared with the corresponding controls (anti-β_2GPI/β_2GPI complex alone), EGCG (50 μg/ml) decreased the TLR4 mRNA

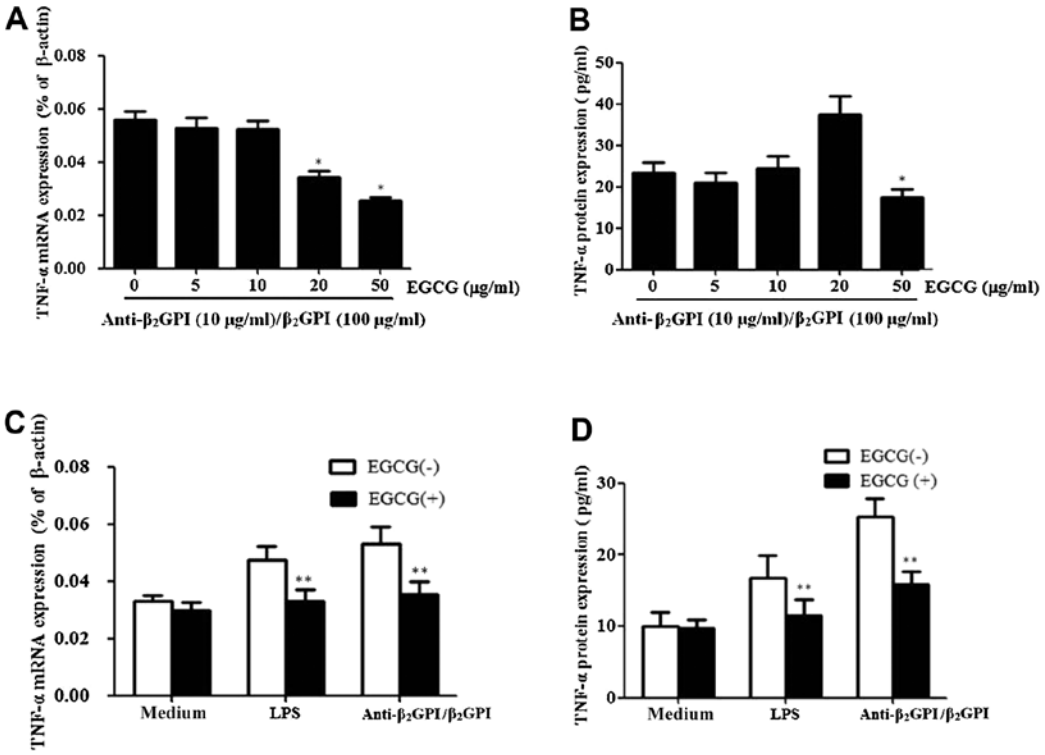

Figure 3. Effects of epigallocatechin-3-gallate (EGCG) on the mRNA and protein expression levels of tumor necrosis factor-α (TNF-α) in THP-1 cells. Cells ($2x10^6$) were pre-treated with or without the indicated concentrations of EGCG for 1 h and then incubated with anti-β_2-glycoprotein I (β_2GPI) (10 μg/ml)/β_2GPI (100 μg/ml) complex for 2 or 24 h, as indicated. On the other hand, other THP-1 cells ($2x10^6$) were incubated with anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex or lipopolysaccharide (LPS) (500 ng/ml) in the absence or presence of EGCG (50 μg/l) for the indicated periods of time. EGCG was not removed from the medium. Total RNA was collected from the cells (2 h) or cultural supematants (24 h). (A and C) TNF-α mRNA and (B and D) protein expression was detected by qRT-PCR and ELISA kits. Data are presented as the means ± SEM from 3 independent experiments. *P<0.05 vs. cells treated with medium only (medium); **P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone.

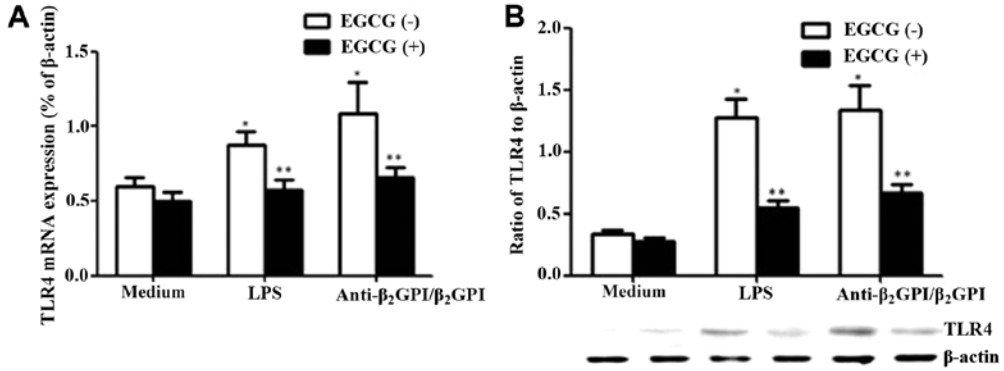

Figure 4. Effects of epigallocatechin-3-gallate (EGCG) on Toll-like receptor 4 (TLR4) expression in THP-1 cells. Cells ($2x10^6$) were stimulated with anti-β_2-glycoprotein I (β_2GPI) (10 μg/ml)/β_2GPI (100 μg/ml) complex or lipopolysaccharide (LPS) (500 ng/ml) in the absence or presence of EGCG (50 μg/ml) for 2 or 6 h. EGCG was not removed from the medium. Total RNA of cells (2 h) and cell lysates(6 h) were collected for the measurement of (A) TLR4 mRNA and (B) protein levels using qRT-PCR and western blot analysis, respectively. Data are presented as the means ± SEM from 3 independent experiments. *P<0.05 vs. cells treated with medium alone (medium); **P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone.

and protein expression levels by approximately 40 and 50%, respectively (P>0.05). EGCG alone had no significant effect on TLR4 expression compared to treatment with medium alone.

Effect of EGCG on MAPK signaling pathways. Previously, we found that the anti-β_2GPI/β_2GPI complex or LPS stimulated the activation of MAPK pathways in THP-1 cells within 30 min of treatment (11). In this study, we further investigated whether EGCG suppresses the activation of MAPKs induced by the anti-β_2GPI/β_2GPI complex or LPS in THP-1 cells. The anti-β_2GPI/β_2GPI and LPS significantly increased the phosphorylation of p38 MAPK (p-p38), ERK1/2 (p-ERK) and JNK1/2 (p-JNK) in the cells (P<0.05 vs. medium only) (Fig. 5). As the concentration of EGCG increased (0-50 μg/ml), the expression levels of total p38 MAPK (t-p38), ERK1/2 (t-ERK) and JNK1/2 (t-JNK) were not altered, but the levels of p-p38 (Fig. 5A) and p-ERK1/2 (Fig. 5B) in the THP-1 cells stimulated with the anti-β_2GPI/β_2GPI complex gradually

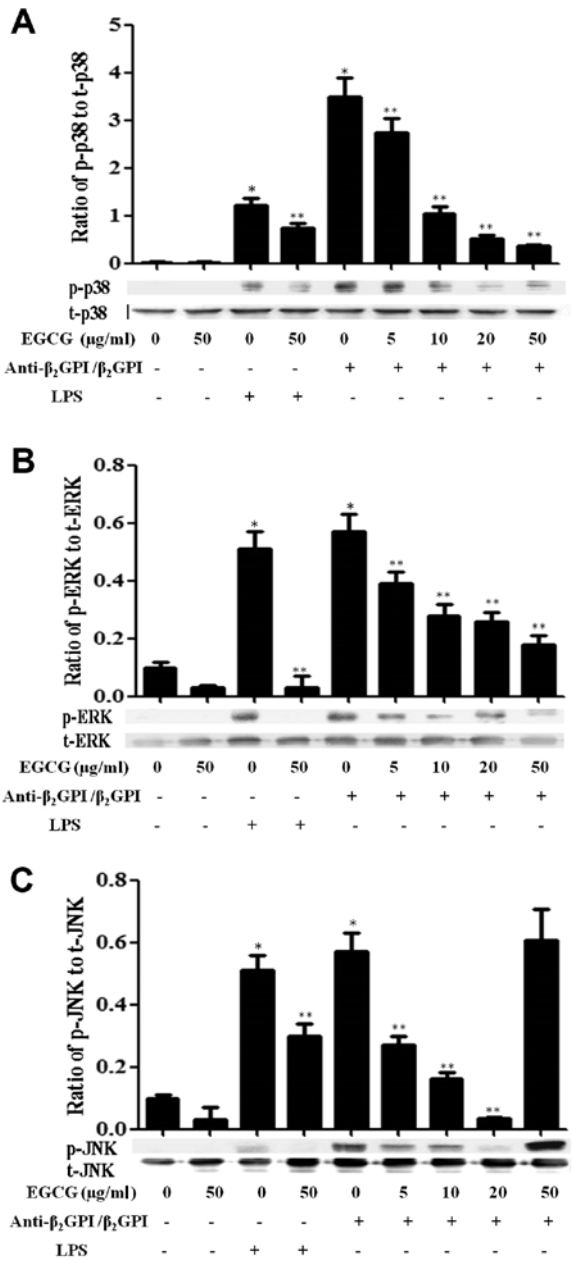

Figure 5. Effects of epigallocatechin-3-gallate (EGCG) on the activation of mitogen-activated protein kinase (MAPK) pathways in THP-1 cells. Cells ($2x10^6$) were pre-treated with or without the indicated concentrations of EGCG for 1 h and then incubated with anti-β_2-glycoprotein I (β_2GPI) (10 μg/ml)/β_2GPI (100 μg/ml) complex or lipopolysaccharide (LPS) (500 ng/ml) for 30 min. EGCG was not removed from the medium. Cell lysates were collected and subjected to western blot analysis with antibodies against (A) total (t-p38) and phosphorylated p38 (p-p38), (B) total (t-ERK) and phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) (p-ERK) or (C) total (t-JNK) and phosphorylated JNK (p-JNK). Data are presented as the means ± SEM from 3 independent experiments. *P<0.05 vs. cells treated with medium only (medium); **P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone.

decreased, indicating a dose-dependent inhibitory effect of EGCG (P<0.05 vs. anti-β_2GPI/β_2GPI complex alone). On the other hand, EGCG (5-20 μg/ml) inhibited the phosphorylation of JNK induced by the anti-β_2GPI/β_2GPI complex gradually; however, a high concentration of EGCG (50 μg/ml) did not reduce the phosphorylation of JNK in the anti-β_2GPI/β_2GPI complex-stimulated THP-1 cells (Fig. 5C). In addition, the

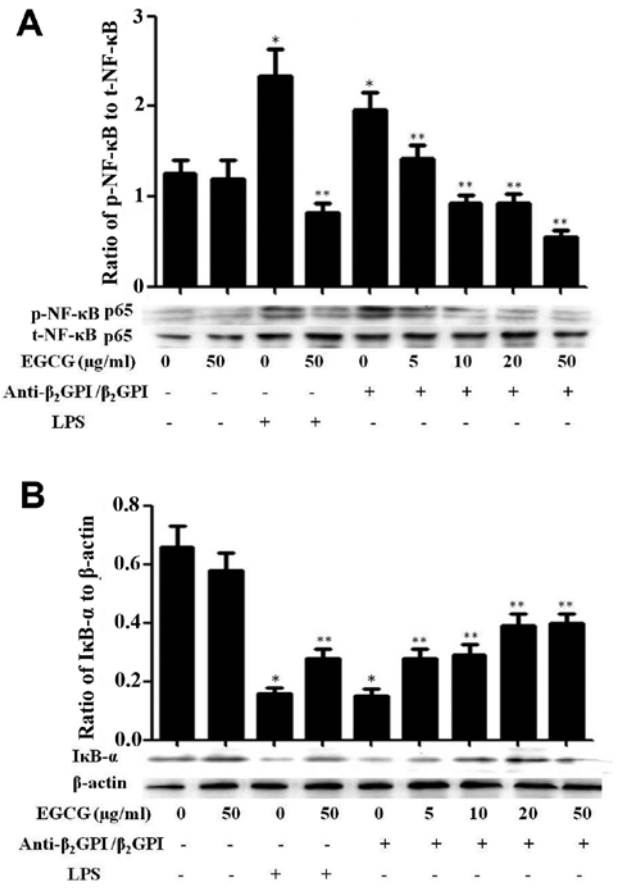

Figure 6. Effects of epigallocatechin-3-gallate (EGCG) on nuclear factor-κB (NF-κB) activation in THP-1 cells. Cells ($2x10^6$) were pre-treated with or without the indicated concentrations of EGCG for 1 h and then incubated with anti-β_2-glycoprotein I (β_2GPI) (10 μg/ml)/β_2GPI (100 μg/ml) complex or lipopolysaccharide (LPS) (500 ng/ml) for 60 min. EGCG was not removed from the medium. Cell lysates were collected and subjected to western blot analysis with antibodies against (A) total and phosphorylated NF-κB, IκB-α and (B) β-actin. Data are presented as the means ± SEM from 3 independent experiments. *P<0.05 vs. cells treated with medium only (medium); **P<0.05 vs. anti-β_2GPI/β_2GPI complex or LPS stimulation alone.

stimulatory effects of LPS (500 ng/ml) on MAPKs, including p-p38, p-ERK1/2 and p-JNK were also blocked by EGCG (50 μg/ml) (P<0.05 vs LPS stimulation alone).

Effect of EGCG on NF-κB activation in THP-1 cells. NF-κB, originally emerged as a major regulator of innate and adaptive immunity and inflammatory responses, and has been shown to be involved in the signal transduction of TLR4/LPS (20). In a previous study, we indicated that NF-κB can be activated and plays important roles in the process of anti-β_2GPI/β_2GPI-induced TF expression in THP-1 cells, thereby contributing to the pathological processes of APS (9). In this study, we further investigated the effects of EGCG on the activation of the NF-κB pathway in THP-1 cells. Treatment with anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex or LPS (500 ng/ml) considerably increased the phosphorylation of NF-κB, which was inhibited by pre-treatment with EGCG (5-50 μg/ml) in a dose-dependent manner (Fig. 6A). On the other hand, treatment with the anti-β_2GPI (10 μg/ml)/β_2GPI (100 μg/ml) complex or LPS (500 ng/ml) effectively inhibited the expression of IκB-α (inhibitor of NF-κB) in THP-1 cells and this inhibitory effect

was reversed by EGCG (5-50 μg/ml) pre-treatment in a dose-dependent manner (Fig. 6B). Similarly, the stimulatory effects of LPS (100 ng/ml) on NF-κB were also blocked by EGCG (50 μg/ml). EGCG alone had no significant effect on the NF-κB pathway compared to treatment with medium alone. These results suggest that EGCG blocks the transduction of the NF-κB pathway induced by the anti-β_2GPI/β_2GPI complex.

Discussion

APS is defined by one or more episodes of thrombosis or unexplained pregnancy loss in association with persisting positive aPLs, either anticardiolipin (aCL), anti-β_2GPI, and/or lupus anticoagulant (LAC) (21). APS that is frequently associated with underlying autoimmune disorders, most commonly systemic lupus erythematosus (SLE), is known as secondary APS, otherwise it is identified as primary APS. In its most severe and life-threatening form, APS is termed as catastrophic APS. Since the identification of APS by Hughes in 1985, the mechanisms or targets of injury underlying thrombotic events have been vigorously debated. aPL antibodies, which bind to a range of cellular targets, such as platelets, monocytes or/and endothelial cells, can upregulate TF. TF as the main initiation factor of the blood coagulation cascade, has been suggested to be a main potential mechanism of APS-related thrombosis (22). On the other hand, the enhanced expression and secretion of cytokines, particularly TNF-α, may contribute to the thrombotic activity in patients with APS (23). In the present study, we first demonstrate that the anti-β_2GPI/β_2GPI complex or LPS activate the monocytic THP-1 cell line, by increasing TF and TNF-α expression (Fig. 1). The current finding suggests that the anti-β_2GPI/β_2GPI complex can induce pro-inflammatory and procoagulant phenotypes characterized by the release of TNF-α and TF, respectively.

Recent improvements in the understanding of the pathogenic mechanisms of APS, including the aPL-induced activation of platelets, endothelial cells, monocytes, complement and coagulation cascade, has led to the identification of potential targets and future therapies for APS. In general, treatment regimens for APS must be individualized according to the current clinical status of the patient and the history of thrombotic events. Low-dose aspirin is used widely in the treatment of patients with APS. However, the effectiveness of low-dose aspirin as the primary prevention therapy for APS remains unproven. Clopidogrel has anecdotally been reported to be helpful in individuals with APS and may be useful in patients allergic to aspirin (24). In patients with SLE, hydroxychloroquine is also considered to be helpful as it has intrinsic antithrombotic properties (25). If thrombotic events reoccur in patients with APS, a combination of warfarin and aspirin may be used (26). Treatment for significant thrombotic events in patients with APS is generally lifelong. Current management strategies for patients with APS are restricted mainly to anticoagulation therapy, which is not effective in all patients (27). Despite antithrombotic therapy, up to 30% of patients with APS have recurrent thrombotic events (28). Furthermore, patients (2-3%)may experience bleeding complications with conventional anticoagulation therapy (29). The optimal treatment strategy for patients with APS resistant or intolerant to long-term anticoagulation remains to be discovered.

Based on recent advances in the pathophysiology of APS, many new therapeutic modalities for treating and/or preventing thrombosis in patients with APS have been reported, such as B-cell targeted therapies (30), eaulizumab (a monoclonal antibody directed against complement C5) (31), defibrotide (an adenosine receptor against that blocks monocyte TF expression) (31), statins (32), antiplatelet agents (33) and intracellular pathway inhibitors [SB203580, a specific inhibitor of p38 MAPK (34), MG132, a specific inhibitor of NF-κB (35)]. However, available data on humans are limited to support these innovative approaches.

Green tea, produced from the tea plant *Camellia sinensis*, has been consumed as a popular beverage worldwide for thousands of years. The most significant phytochemical in green tea is a polyphenol termed EGCG. EGCG has been the subject of interest in a number of studies investigating its potential use as a therapeutic agent for a broad range of disorders over the past few decades. For example, EGCG (1-30 μM) has been shown to inhibit TNF-α and histamine induced endothelial TF expression and activity in a concentration dependent manner, resulting in a 87% reduction in TF expression (36). EGCG has been shown to have an enhanced inhibitory effect on the release of TNF-α from BALB/3T3 cells treated with okadaic acid (37). EGCG has also been shown to inhibit the production of inflammatory mediators, such as TNF-α, IL-6 and IL-8, through the inhibition of intracellular Ca^{2+} levels (38). These results are generally in accordance with those in our current study. However, to our knowledge, EGCG has never been investigated as a therapeutic strategy for APS. As demonstrated in this study, the anti-β_2GPI/β_2GPI complex-enhanced TF and TNF-α mRNA expression, as well as TF activity were inhibited in a dose-dependent manner by EGCG. The maximal inhibition rates of EGCG (50 μg/ml) on TF (mRNA level and activity) and TNF-α expression (mRNA and protein) (induced by anti-β_2GPI/β_2GPI complex) were approximately 48, 50, 51 or 30%, respectively and were comparable to those of EGCG on LPS-induced expression (Figs. 2 and 3). However, it is puzzling that EGCG (0-20 μg/ml) had irregular effects on the TNF-α levels of cells stimulated with the anti-β_2GPI/β_2GPI complex. Our study demonstrated that EGCG may be effective in preventing thrombosis in patients with APS. However, the biological mechanisms of action of EGCG and its use as a primary prevention treatment for APS remain unproven.

In our previous studies, we revealed that TLR4 acts as a co-factor for Annexin A2 on the THP-1 cell surface, and contributes to anti-β_2GPI/β_2GPI complex-enhanced TF expression in THP-1 cells (6,8,16,17). In addition, MD-2 and MyD88 are also upregulated and cooperate with TLR4, thus leading to the activation of MAPKs in this process (11). We further demonstrated that the anti-β_2GPI/β_2GPI complex upregulated TF expression in THP-1 cells, following the activation of nuclear transcription factors, including NF-κB and AP-1 (9). In addition, the intracellular signal transduction pathway of TLRs-MAPKs-NF-κB/AP-1 axis in anti-β_2GPI/β_2GPI complex-induced TF and TNF-α expression may contribute to the pathological mechanisms responsible for causing thrombosis in patients with APS.

In a previous study, EGCG was reported to affect an array of signal pathways through which it exerts its pharmacological activities. EGCG was shown to inhibit the degradation of IRAK induced by IL-1β in A549 cells (39). This polyphenol

has also been shown to inhibit the LPS-induced activation of MAPK pathways, including ERK1/2, p38 and JNK (40). It has been described that EGCG suppresses the LPS-induced activation of NF-κB by blocking the degradation of IκB-α following IκB-α phosphorylation (41). Moreover, EGCG inhibits MyD88-dependent signaling pathways and TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent signaling pathways of TLRs in RAW264.7 cells, which suppresses inflammatory responses (42).

In the present study, we further demonstrate that EGCG (50 μg/ml) significantly suppresses TLR4 mRNA and protein expression in cells stimulated with the anti-β_2GPI/β_2GPI complex or LPS (Fig. 4). Our results suggest that EGCG is capable of blocking the expression of TLR4 in THP-1 cells and therefore, can contribute to the suppression of THP-1 cell activation by inhibiting TF and TNF-α expression. As shown by our results, the anti-β_2GPI/β_2GPI complex or LPS activated MAPK pathways, as indicated by the increased phosphorylation of p38, ERK1/2 and JNK, as well as by the NF-κB (p65/RelA) pathway in the nuclear fraction (Figs. 5 and 6). Furthermore, EGCG (5-50 μg/ml) inhibited both MAPK (including p38, ERK1/2 as well as JNK) and NF-κB activation induced by the anti-β_2GPI/β_2GPI complex in a dose-dependent manner. Similarly, EGCG at a concentration of 50 μg/ml markedly inhibited LPS-induced MAPK and NF-κB activation. These results indicate that the blockade of MAPK and NF-κB activation is the major mechanism responsible for the inhibitory effects of EGCG on the anti-β_2GPI/β_2GPI complex-mediated expression of TF and TNF-α. However, it is worth mentioning that EGCG (5-10 μg/ml) only slightly inhibited TF and TNF-α expression, but significantly blocked the activation of MAPKs and NF-κB. This may explain the fact that the anti-β_2GPI/β_2GPI complex stimulated cells not only throught he MAPKs/NF-κB pathway, but also through other receptors. Zhang *et al* (43) recently demonstrated that WNT signaling activation stimulates the production of the pro-inflammatory cytokines, IL-18 and TNF-α, in the spinal cord, suggesting that it may contribute to the uptake of the anti-β_2GPI/β_2GPI complex.

In conclusion, data from our present study, as well as from our previous studies, strongly indicate that EGCG inhibits the anti-β_2GPI/β_2GPI-induced activation of THP-1 cells by decreasing TF and TNF-α expression levels via blocking the intracellular signal transduction pathway of TLRs-MAPKs-NF-κB axis, and may serve as a preventive and therapeutic agent for APS.

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References

1. de Groot PG and Derksen RH: Antiphospholipid antibodies: update on detection pathophysiology, and treatment. Curr Opin Hematol 11: 165-169, 2004.
2. Miyakis S, Lockshin MD, Atsumi T, *et al*: International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 4: 295-306, 2006.
3. Bas de Laat H, Derksen RH and de Groot PG: beta2-glycoprotein I, the playmaker of the antiphospholipid syndrome. Clin Immunol 112: 161-168, 2004.
4. Mulla MJ, Brosens JJ, Chamley LW, *et al*: Antiphospholipid antibodies induce a pro-inflammatory response in first trimester trophoblast via the TLR4/MyD88 pathway. Am J Reprod Immunol 62: 96-111, 2009.
5. Mehdi AA, Uthman I and Khamashta M: Antiphospholipid syndrome: pathogenesis and a window of treatment opportunities in the future. Eur J Clin Invest 40: 451-464, 2010.
6. Xie H, Zhou H, Wang H, Chen D, Xia L, Wang T and Yan J: Anti-β(2)GPI/β(2)GPI induced TF and TNF-α expression in monocytes involving both TLR4/MyD88 and TLR4/TRIF signaling pathways. Mol Immunol 53: 246-254, 2013.
7. Lu YC, Yeh WC and Ohashi PS: LPS/TLR4 signal transduction pathway. Cytokine 42: 145-151, 2008.
8. Zhou H, Yan Y, Xu G, *et al*: Toll-like receptor (TLR)-4 mediates anti-β(2) GPI/β(2) GPI-induced tissue factor expression in THP-1 cells. Clin Exp Immunol 163: 189-198, 2011.
9. Xia L, Zhou H, Hu L, *et al*: Both NF-κB and c-Jun/AP-1 involved in anti-β_2GPI/β_2GPI-induced tissue factor expression in monocytes. Thromb Haemost 109: 643-651, 2013.
10. Lawrence MC, Jivan A, Shao C, *et al*: The roles of MAPKs in disease. Cell Res 18: 436-442, 2008.
11. Zhou H, Chen D, Xie H, Xia L, Wang T, Yuan W and Yan J: Activation of MAPKs in the anti-β_2GPI/β_2GPI-induced tissue factor expression through TLR4/IRAKs pathway in THP-1 cells. Thromb Res 130: e229-e235, 2012.
12. Lee SH, Nam HJ, Kang HJ, Kwon HW and Lim YC: Epigallocatechin-3-gallate attenuates head and neck cancer stem cell traits through suppression of Notch pathway. Eur J Cancer 49: 3210-3218, 2013.
13. Sarkar FH, Li Y, Wang Z and Kong D: The role of nutraceuticals in the regulation of Wnt and Hedgehog signaling in cancer. Cancer Metastasis Rev 29: 383-394, 2010.
14. Lin HY, Hou SC, Chen SC, *et al*: (-)-Epigallocatechin gallate induces Fas/CD95-mediated apoptosis through inhibiting constitutive and IL-6-induced JAK/STAT3 signaling in head and neck squamous cell carcinoma cells. J Agric Food Chem 60: 2480-2489, 2012.
15. Kim SJ, Jeong HJ, Lee KM, *et al*: Epigallocatechin-3-gallate suppresses NF-kappaB activation and phosphorylation of p38 MAPK and JNK in human astrocytoma U373MG cells. J Nutr Biochem 18: 587-596, 2007.
16. Zhou H, Wolberg AS and Roubey RA: Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. Blood 104: 2353-2358, 2004.
17. Zhou H, Wang H, Li N, Yu Y, Huang H, Yan Y and Wang T: Annexin A2 mediates anti-β_2GPI/β_2GPI-induced tissue factor expression on monocytes. Int J Mol Med 24: 557-562, 2009.
18. Xu G, Wen H, Zhou H, *et al*: Involvement of IRAKs and TRAFs in anti-β_2GPI/β_2GPI-induced tissue factor expression in THP-1 cells. Thromb Haemost 106: 1158-1169, 2011.
19. Pierangeli SS, Vega-Ostertag ME, Raschi E, *et al*: Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. Ann Rheum Dis 66: 1327-1333, 2007.
20. Vallabhapurapu S and Karin M: Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 27: 693-733, 2009.
21. McIntyre JA, Wagenknecht DR and Faulk WP: Antiphospholipid antibodies: discovery, definitions, detection and disease. Prog Lipid Res 42: 176-237, 2003.
22. Boles J and Mackman N: Role of tissue factor in thrombosis in antiphospholipid antibody syndrome. Lupus 19: 370-378, 2010.
23. Swadzba J, Iwaniec T and Musial J: Increased level of tumor necrosis factor-α in patients with antiphospholipid syndrome: marker not only of inflammation but also of the prothrombotic state. Rheumatol Int 31: 307-313, 2011.
24. Agaba AE, Charaklias N, Babu-Victor A, *et al*: Antiphospholipid syndrome: a series of surgical emergencies and the current evidence for its managemen. Ann R Coll Surg Engl 88: 370-374, 2006.
25. Petri M: Use of hydroxychloroquine to prevent thrombosis in systemic lupus erythematosus and in antiphospholipid antibody-positive patients. Curr Rheumatol Rep 13: 77-80, 2011.
26. Les I, Guillermo RL, Munther A, *et al*: Intensity and duration of anticoagulation therapy in antiphospholipid syndrome. Semin Thromb Hemost 38:339-347, 2012.
27. Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GL and Harris EN: Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 99: 1997-2002, 1999.

28. Gharavi AE, Pierangeli SS, Colden-Stanfield M, Liu XW, Espinola RG and Harris EN: GDKV-induced antiphospholipid antibodies enhance thrombosis and activate endothelial cells in vivo and in vitro. J Immunol 163: 2922-2927, 1999.
29. Pierangeli SS, Liu SW, Anderson G, Barker JH and Harris EN: Thrombogenic properties of murine anti-cardiolipin antibodies induced by beta 2 glycoprotein 1 and human immunoglobulin G antiphospholipid antibodies. Circulation 94: 1746-1751, 1996.
30. Iglesias Jiménez E, Camacho-Lovillo M, Falcón-Neyra D, Lirola-Cruz J and Neth O: Infant with probable catastrophic antiphospholipid syndrome successfully managed with rituximab. Pediatrics 125: e1523-e1528, 2010.
31. Corbacioglu S, Cesaro S, Faraci M, *et al*: Defibrotide for prophylaxis of hepatic veno-occlusive disease in paediatric haemopoietic stem-cell transplantation: an open-label, phase 3, randomised controlled trial. Lancet 379: 1301-1309, 2012.
32. Espinosa G, Berman H and Cervera R: Management of refractory cases of catastrophic antiphospholipid syndrome. Autoimmun Rev 10: 664-668, 2011.
33. Forastiero RR, Martinuzzo ME and de Larrañaga GF: Circulating levels of tissue factor and proinflammatory cytokines in patients with primary antiphospholipid syndrome or leprosy related antiphospholipid antibodies. Lupus 14: 129-136, 2005.
34. Yoon KH: Sufficient evidence to consider hydroxychloroquine as an adjunct therapy in antiphospholipid antibody (Hughes') syndome. J Rheumatol 29: 1574-1575, 2002.
35. Montiel-Manzano G, Romay-Penabad Z, Papalardo de Martínez E, Meillon-García LA, García-Latorre E, Reyes-Maldonado E, Pierangeli SS: In vivo effects of an inhibitor of nuclear factor-kappa B on thrombogenic properties of antiphospholipid antibodies. Ann NY Acad Sci 1108: 540-553, 2007.
36. Holy EW, Stämpfli SF, Akhmedov A, *et al*: Laminin receptor activation inhibits endothelial tissue factor expression. J Mol Cell Cardiol 48: 1138-1145, 2010.
37. Suganuma M, Sueoka E, Sueoka N, *et al*: Mechanisms of cancer prevention by tea polyphenols based on inhibition of TNF-α expression. Biofactors 13: 67-72, 2000.
38. Shin HY, Kim SH, Jeong HJ, *et al*: Epigallocatechin-3-gallate inhibits secretion of TNF-alpha, IL-6 and IL-8 through the attenuation of ERK and NF-kappaB in HMC-1 cells. Int Arch Allergy Immunol 142: 335-344, 2007.
39. Wheeler DS, Catravas JD, Odoms K, Denenberg A, Malhotra V and Wong HR: Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinflammatory signal transduction in cultured respiratory epithelial cells. J Nutr 134: 1039-1044, 2004.
40. Lin YL and Lin JK: (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. Mol Pharmacol 52: 465-472, 1997.
41. Ahn SC, Kim GY, Kim JH, *et al*: Epigallocatechin-3-gallate, constituent of green tea, suppresses the LPS-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and NF-kappaB. Biochem Biophys Res Commun 313: 148-155, 2004.
42. Youn HS, Lee JY, Saitoh SI, Miyake K, Kang KW, Choi YJ and Hwang DH: Suppression of MyD88- and TRIF-dependent signaling pathways of Toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. Biochem Pharmacol 72: 850-859, 2006.
43. Zhang YK, Huang ZJ, Liu S, Liu YP, Song AA and Song XJ: WNT signaling underlies the pathogenesis of neuropathic pain in rodents. J Clin Invest 123: 2268-2286, 2013.