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RESEARCH ARTICLE

# Anti-$\beta_2$GPI/$\beta_2$GPI complexes induce platelet activation and promote thrombosis via p38MAPK: a pathway to targeted therapies

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**Abstract** **Anti-$\beta_2$ glycoprotein I (anti-$\beta_2$GPI) antibodies are important contributors to the development of thrombosis. Anti-$\beta_2$GPI antibody complexes with $\beta_2$GPI are well known to activate monocytes and endothelial cells via the intracellular NF-κB pathway with prothrombotic implications. By contrast, the interaction of anti-$\beta_2$GPI/$\beta_2$GPI complexes with platelets has not been extensively studied. The p38 mitogen-activated protein kinase (MAPK) pathway has been recognized to be an important intracellular signaling pathway in the coagulation cascade and an integral component of arterial and venous thrombosis. The present study reveals that levels of anti-$\beta_2$GPI/$\beta_2$GPI complexes in sera are positively associated with p38MAPK phosphorylation of platelets in thrombotic patients. Furthermore, SB203580 inhibits anti-$\beta_2$GPI/$\beta_2$GPI complex-induced platelet activation. Thrombus formation decreased in *p38MAPK*$^{-/-}$ mice after treatment with anti-$\beta_2$GPI/$\beta_2$GPI complexes. In conclusion, p38MAPK may be a treatment target for anti-$\beta_2$GPI antibody-associated thrombotic events.**

**Keywords** anti-$\beta_2$GPI antibody; $\beta_2$GPI; platelet; p38MAPK; thrombosis; complex

## Introduction

Severe thrombosis and its ischemic consequences are major worldwide health issues. Given emerging links between autoimmune disorders and thrombosis, a compelling reason to further explore anti-$\beta_2$GPI antibody/hypercoagulable state interplay is now available. Anti-$\beta_2$GPI antibodies are a common acquired risk factor in antiphospholipid syndrome, which is found in association with arterial or venous thrombosis [1]. Many studies have previously demonstrated that anti-$\beta_2$GPI antibodies promote the formation of platelet-rich thrombi [2], which suggests that the effect of anti-$\beta_2$GPI antibodies on platelets may be directly pathogenic in thrombosis. Despite extensive studies of the aforementioned cell types, the interaction of anti-$\beta_2$GPI/$\beta_2$GPI complexes with the most thrombosis-promoting cells in human blood, namely, platelets, has only rarely been considered [3–7].

p38 mitogen-activated protein kinase (MAPK) is a core trigger of blood coagulation because of its important role in the regulation of many intracellular responses, such as migration, proliferation, differentiation, and apoptosis [8–10]. Current evidence suggests that p38MAPK is an attractive drug target for neoplastic and autoimmune and inflammatory diseases [11]. While the role of p38MAPK phosphorylation in anti-$\beta_2$GPI/$\beta_2$GPI complex-stimulated platelet activation and thrombosis is unclear, a receptor-dependent pathway may be involved. Since our preliminary study showed that anti-$\beta_2$GPI/$\beta_2$GPI complexes induce platelet activation *in vitro* via both apoER2′ and GPIbα receptors [12], we further systematically investigated the detailed mechanisms underlying p38MAPK-mediated platelet activation and thrombosis *in vitro* and *in vivo*.

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## Materials and methods

### Reagents

Anti-$\beta_2$GPI antibody and $\beta_2$GPI were purchased from Sino Biological Inc. (Beijing, China). Human IgG, thrombin, adenosine diphosphate, and SB203580 were from Beyotime (Shanghai, China). Primary antibodies against phosphorylated-p38MAPK and β-actin, as well as peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-human secondary antibodies, were purchased from Cell

Signaling Technology (Beverly, MA, USA). Antibodies against CD62P, CD41, and PAC-1 were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). TAK-242 and Fc-blocking reagents were purchased from Sigma (St. Louis, MO, USA), and the semi-quantitative enzyme-linked immunosorbent kit was obtained from Euroimmun (Lubeck, Germany).

### Patients

Twenty patients with clinical or radiological evidence of thrombus were enrolled; of these, 11 patients tested positive for anti-$\beta_2$GPI IgG antibody by ELISA. Additionally, 10 patients with no thrombus but who were anti-$\beta_2$GPI IgG antibody-positive were included, along with 15 healthy volunteers who had no autoimmune disease and had received no medication for at least 10 days prior to the experiment. The clinical characteristics of these patients are described in Table 1. Samples from the patients were collected with the approval of the Harbin Medical University Institutional Ethics Committee, and informed consent was obtained in accordance with the *Declaration of Helsinki*. After collection, all patient samples were numbered and de-identified to maintain patient confidentiality.

### Animals

Control wild-type (WT) mice were obtained from Harbin Medical University Laboratory Animal Center, which were purchased from Kunming, China, and *p38MAPK*$^{-/-}$ mice were purchased from Jackson Laboratories. All mice used in this study were 8–10 weeks of age when the experiments were initiated. Approval for animal experimental studies was received from the Institutional Animal Care and Use Committee of Harbin Medical University. All surgical procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

### Human platelet preparation and stimulation

For studies using human platelets, fresh blood was drawn via venipuncture from healthy adult volunteers and patients. Blood was collected in sodium citrate anticoagulant. Platelet-rich plasma (PRPs) were obtained via centrifugation for 10 min at 250 × *g* at 25 °C. Then, the PRPs were centrifuged at 1500 × *g* for 10 min at 25 °C and resuspended in Tyrode's buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl, and 12 mmol/L $NaHCO_3$, pH 7.4, 5 mmol/L glucose), adjusted to 2 × $10^8$ cells/mL. Control human platelet samples (0.2–0.5 mL) were stimulated with the anti-$\beta_2$GPI/$\beta_2$GPI complexes (10/100 μg/mL) or 20 μmol/L thrombin for 30 min. In some experiments, the platelets were pre-incubated with an Fc receptor blocker (20 μL), 20 μmol/L SB203580, or 10 μmol/L TAK-242 within 30 min before the complexes were incubated.

### Western blot analysis

Western blot was conducted according to previously described methods [3]. The membranes were incubated with primary antibodies against human β-actin (1:100) or

**Table 1** Characteristics of the patients

| Variable | Control group | Anti-$\beta_2$GPI antibodies ( + ) patients | | Anti-$\beta_2$GPI antibody (−) thrombotic patients |
|---|---|---|---|---|
| | | No thrombus | Thrombotic | |
| *N* | 15 | 10 | 11 | 9 |
| Mean age-years | 40±5 | 35±2 | 37±5 | 45±3 |
| Female sex-no. (%) | 5 (33.3) | 7 (70.0) | 4 (36.4) | 3 (33.3) |
| Platelets | (254±22) × $10^9$/L | (225±35) × $10^9$/L | (304±52) × $10^9$/L | (284±40) × $10^9$/L |
| Venous thromboembolic-no. | – | – | 3 | 5 |
| Pulmonary embolism-no. (%) | – | – | 1 (33.3) | 3 (60.0) |
| Deep vein thrombosis-no. (%) | – | – | 2 (66.7) | 2 (40.0) |
| Arterial thromboembolic-no. | – | – | 8 | 4 |
| Stroke-no. (%) | – | – | 4 (50.0) | 2 (50.0) |
| Acute myocardial infarction-no. (%) | – | – | 3 (37.5) | 1 (25.0) |
| Renal artery thrombosis-no. (%) | – | – | 1 (12.5) | 1 (25.0) |
| No thrombosis | – | – | – | – |
| SLE-no. (%) | – | 5 (50.0) | – | – |
| Viral meningitis-no. (%) | – | 2 (20.0) | – | – |
| Cerebral hemorrhage-no. (%) | – | 3 (30.0) | – | – |

SLE: systemic lupus erythematosus. Data are presented as mean±SEM unless otherwise noted. ELISA results were reported semi-quantitatively in standard IgG anti-$\beta_2$GPI units as follows: positive (IgG, >20). Platelet numbers were tested by an XE-5000 automated hematology analyzer.

rabbit phosphorylated-p38MAPK (1:1000) overnight at 4 °C. Following 3 washes with Tris-Buffered Saline Tween (TBST), the membranes were incubated with HRP-conjugated goat anti-human/rabbit secondary antibodies for 1 h at 37 °C. Finally, the immunoblots were developed using ECL Western blot detection reagents, imaged, and quantitated using a Bio-Rad Fluor-S MultiImager (CHAMGTE15500).

**ELISA analysis**

The concentration of the anti-$\beta_2$GPI/$\beta_2$GPI immune complexes was detected by Biasiolo [13]. Exactly 100 μL of rabbit anti-human $\beta_2$GPI IgG (1.5 mg/mL in phosphate-buffered saline, PBS) was adsorbed onto a polyvinylchloride plate overnight at 4 °C. Then, 100 μL of a 1:10 dilution of patient plasma and control plasma was added to the plates, and incubation was conducted for another 1 h; 100 μL of $\beta_2$GPI (1 mg/mL) and a control IgG (0.2 mg/mL) served as a negative control, while 100 μL of *in vitro* generated immune complexes (1 mg/mL) was used as a positive reference control. Following six washes, the bound complexes were detected using 100 μL phosphatase-conjugated goat anti-mouse IgG (1:10 000) as a positive control, 100 μL alkaline phosphatase-specific goat anti-human IgG as a negative control, and sample plasmas. After incubation (1 h) followed by six washes in PBS, 100 μL of freshly prepared *p*-nitrophenylphosphate solution was added to each well. The color reaction was then measured at 405 nm. The results are expressed as the mean value of three replicates.

**Flow cytometry analysis**

Human platelets were treated with phycoerythrin (PE)-labeled anti-human P-selectin (CD62P) and FITC-labeled anti-human GPIIb/IIIa complex (PAC-1) antibodies. Platelets were analyzed via flow cytometry using a FACS Calibur instrument (BD Biosciences, USA). Antibody binding was determined as the percentage of positive platelets over a fluorescence threshold gated over a platelet population stained with the isotype-matched control IgG. The experiments were conducted and analyzed in biological triplicates.

**Confocal microscopy analysis**

Platelets were rapidly fixed with a buffered 4% paraformaldehyde solution. After 10 min, the aldehyde was removed with successive washes using PBS (pH 7.4). For laser scanning confocal microscopic analysis, specimens were incubated with CD62P and FITC-labeled anti-human CD41. High-resolution confocal reflection microscopy was performed using an Olympus FluoView FV1000 laser-scanning confocal microscope equipped with a 60 × 1.42 NA oil objective to view the platelet microparticles. An Olympus FVS-PSU/IX2-UCB camera and scanning unit and Olympus FluoView and FV1000 image acquisition software (version 5.0) were used for recording.

**Platelet aggregation**

Platelet aggregation was measured in a turbidimetric platelet aggregometer (Helena, USA) at 37 °C with stirring (250 g), and ADP ($10^{-2}$ mol/L) was used as the inducer. The human platelets ($3 \times 10^8$ cells/ L) were washed in modified Tyrode's buffer, and the experiments were repeated at least 3 times.

***In vivo* platelet preparation**

WT and *p38MAPK*$^{-/-}$ mice weighting 22 ± 2 g were intravenously injected with IgG (1 mg/kg) or anti-$\beta_2$GPI/$\beta_2$GPI (1 mg/mL :10 mg/mL) complexes for 15 min. The mice were anesthetized via i.p.-injection with sodium pentobarbital (40 mg/kg body weight). To investigate the effect of the anti-$\beta_2$GPI/$\beta_2$GPI complexes on platelets, circulating blood cells were removed via *in vivo* whole-body perfusion with sodium citrate through the inferior vena cava. Platelet preparation was performed as previously described.

***In vivo* thrombosis and laser Doppler analysis**

To investigate the effect of the anti-$\beta_2$GPI/$\beta_2$GPI complexes on thrombosis exclusively, 10% $FeCl_3$ was applied to Whatman filter paper (1MM, 3 mm length and 1 mm width) and placed under the carotid artery throughout the surgical procedure. Thereafter, blood flow was continuously monitored using a multi-functional laser Doppler system. The time to occlusion was calculated as the interval between the positioning of the filter paper and stable occlusion (no blood flow for 2 min).

**Hematoxylin–eosin staining**

After stimulation with 10% $FeCl_3$ for 5 min, the injured artery and the control contralateral artery were removed and fixed in 10% formaldehyde overnight. The vessels were embedded in paraffin and cut into longitudinal sections 6 μm in thickness. These samples were stained, mounted, and counted under a light microscope at a magnification of 40×.

**Statistics**

Unless otherwise indicated, the data are presented as the mean ± SEM. Differences between treatments were evaluated by one-way ANOVA. Correlations were tested by Pearson's correlation coefficient. For p38MAPK *in*

*vitro* kinase assays, analyses were performed using Fujifilm Science Laboratory 2003, Image Gauge version 4.22 software (Edison, NJ, USA). GraphPad Prism version 5 software was used for statistical analysis and graphic representation. *P* values less than 0.05 were required for the results to be considered statistically significant.

## Results

### p38MAPK phosphorylation of platelets in anti-$\beta_2$GPI antibody-positive patients

We first measured levels of p38MAPK phosphorylation in platelets from anti-$\beta_2$GPI antibody-positive patients. We found that platelets from antibody-positive patients who suffered from thrombotic diseases showed higher levels of p38MAPK phosphorylation compared with the antibody-negative patients (Fig. 1A). In general, platelets from patients with thrombotic diseases exhibited higher levels of p38MAPK phosphorylation than those obtained from antibody-positive patients without thrombus. Therefore, we compared levels of p38MAPK phosphorylation of platelets between anti-$\beta_2$GPI antibody-negative and antibody-positive thrombotic patients (Fig. 1A). We found a clear increase in p38MAPK phosphorylation in antibody-positive thrombotic patients.

### p38MAPK phosphorylation of platelets is dependent on the presence of the anti-$\beta_2$GPI/$\beta_2$GPI complexes

As shown in Fig. 1, the $OD_{405}$ values for the complexes were significantly higher for anti-$\beta_2$GPI antibody-positive patients (thrombotic: mean value $OD_{405}$ = 0.611, range 0.312–0.881; nonthrombotic: mean value $OD_{405}$ = 0.434, range 0.231–0.653) than for the control and antibody-negative thrombotic patients. Furthermore, circulating anti-$\beta_2$GPI/$\beta_2$GPI complexes showed a positive correlation with p38MAPK phosphorylation in these samples (Fig. 1B). We found that the anti-$\beta_2$GPI/$\beta_2$GPI complexes significantly stimulated p38MAPK phosphorylation of

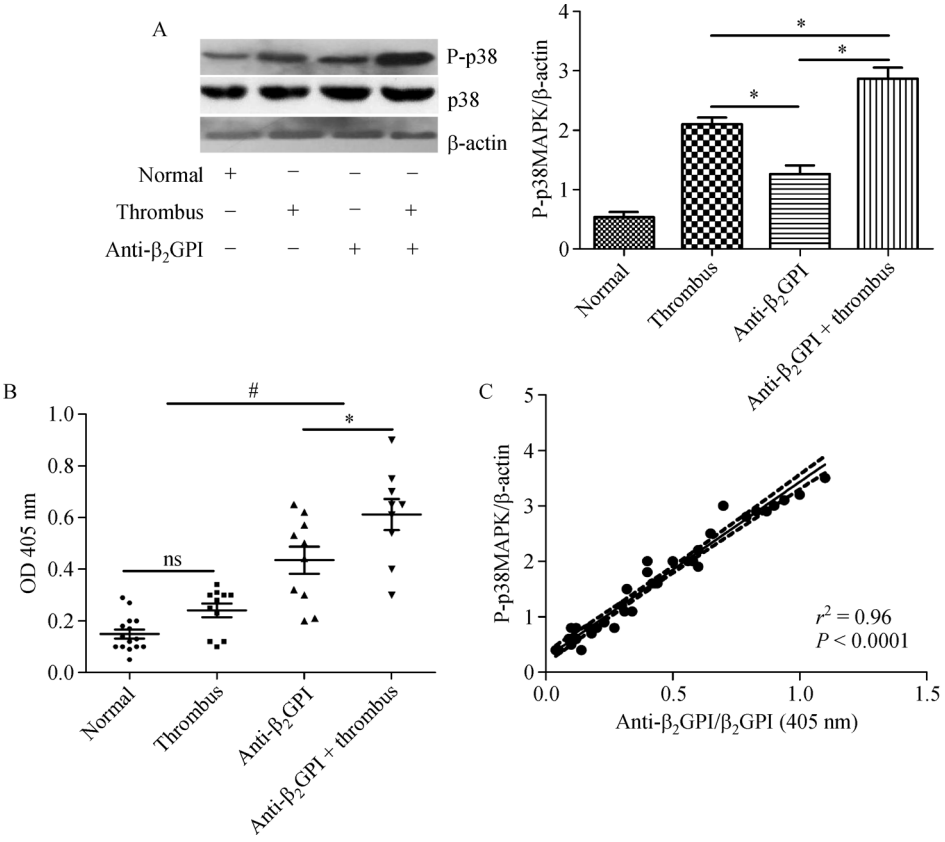


**Fig. 1** p38MAPK activation of platelets in anti-$\beta_2$GPI antibody-positive patients. (A) p38MAPK activation of platelets from anti-$\beta_2$GPI antibody-positive patients with or without thrombus, anti-$\beta_2$GPI antibody-negative thrombotic patients, and healthy controls was determined by Western blot as described in *Materials and methods.* Data are shown as the mean ± SEM and taken from a single experiment representative of three independent experiments. (B) Anti-$\beta_2$GPI/$\beta_2$GPI complexes were measured in thrombotic patients, anti-$\beta_2$GPI antibody-positive patients with or without thrombus, and healthy controls. (C) Correlation between levels of p38MAPK activation and anti-$\beta_2$GPI/$\beta_2$GPI complexes in anti-$\beta_2$GPI antibody-positive patients. $^*P < 0.05$, $^{\#}P < 0.01$, and ns = not significant.

normal platelets compared with the controls and those with anti-$\beta_2$GPI antibodies (Fig. 2A). This effect was independent of the presence of serum. The stimulation persisted even when the Fc region of the anti-$\beta_2$GPI antibody was blocked (Fig. 2B).

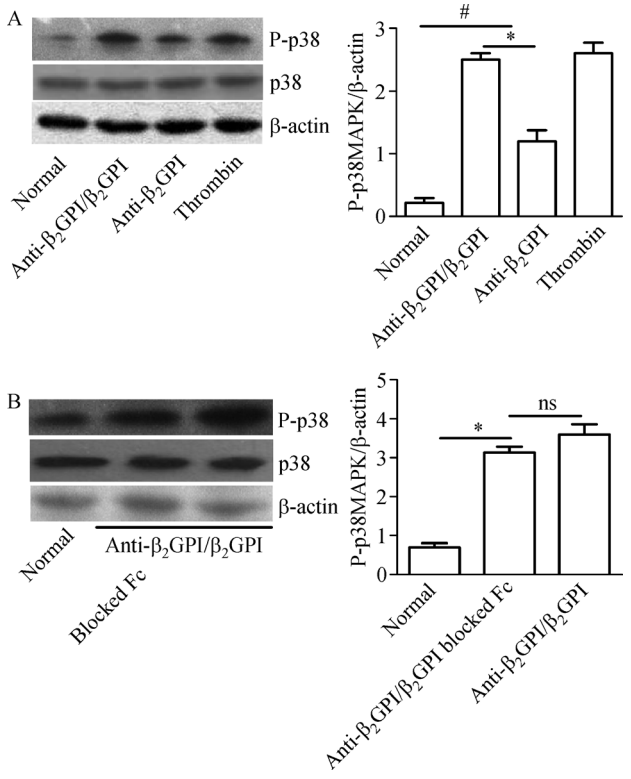


**Fig. 2** p38MAPK activation is associated with the anti-$\beta_2$GPI/$\beta_2$GPI complexes. p38MAPK activation was measured in (A) control platelets stimulated with anti-$\beta_2$GPI/$\beta_2$GPI complexes or anti-$\beta_2$GPI antibodies or in (B) control or Fc receptor-blocked platelets treated with the anti-$\beta_2$GPI/$\beta_2$GPI complexes. Data are shown as the mean ± SEM and taken from a single experiment representative of three independent experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$, and ns = not significant.

## Anti-$\beta_2$GPI/$\beta_2$GPI complex-induced platelet activation is dependent on p38MAPK phosphorylation

We found that the complexes promoted significant GPIIb/IIIa activation and P-selectin expression, which are enhanced after p38MAPK phosphorylation. The p38MAPK inhibitor SB203580 reduced GPIIb/IIIa activation by 12.9% and abolished P-selectin expression by 28.5% compared with the anti-$\beta_2$GPI/$\beta_2$GPI complex group without SB203580 treatment (Fig. 3A). We further examined platelet-derived microparticle (PMP) secretion and platelet aggregation. The levels of microparticle secretion were detected with CD41 and P-selectin. We found that the anti-$\beta_2$GPI/$\beta_2$GPI complexes markedly increased PMP secretion and platelet aggregation compared with the IgG treatment. SB203580 reduced PMP release by 42.3% and prevented platelet aggregation by 14.9% compared with the anti-$\beta_2$GPI/$\beta_2$GPI complex group without SB203580 treatment (Fig. 3B and 3C).

## Anti-$\beta_2$GPI/$\beta_2$GPI complex-mediated p38MAPK phosphorylation and platelet activation are dependent on TLR4

We found that a chemical TLR4 inhibitor, TAK-242, abrogated p38MAPK phosphorylation (Fig. 4A), inhibited platelet GPIIb/IIIa activation by 20% (Fig. 4B), decreased PMP secretion by 18% (Fig. 4C), and weakened aggregation by 17% (Fig. 4D) in response to the anti-$\beta_2$GPI/$\beta_2$GPI complexes compared with those without indicated inhibitor treatment. TAK-242 exerted no significant effect on P-selectin expression (Fig. 4B).

## Anti-$\beta_2$GPI/$\beta_2$GPI complex-enhanced platelet activation and aggregation in mice are dependent on the p38MAPK signaling pathway

We measured p38MAPK phosphorylation in platelets from WT mice (Fig. 5A). Injection of the anti-$\beta_2$GPI/$\beta_2$GPI complexes increased p38MAPK phosphorylation in WT mouse platelets. Therefore, injection of the complexes increased GPIIb/IIIa activation and P-selectin expression of platelets and promoted platelet aggregation. To determine whether platelet activation in mice is dependent on p38MAPK phosphorylation, we injected the anti-$\beta_2$GPI/$\beta_2$GPI complexes into *p38MAPK*$^{-/-}$ mice. *p38MAPK* knockout reduced GPIIb/IIIa activation by 17.7% and P-selectin expression by 38.5% (Fig. 5B) and prevented platelet aggregation by 23.2% compared with platelet from anti-$\beta_2$GPI/$\beta_2$GPI complexes-injected WT mice (Fig. 5C).

## *p38MAPK* knockout can prevent thrombosis of carotid arteries in mice

The effect of anti-$\beta_2$GPI/$\beta_2$GPI complexes on WT mice during the period between $FeCl_3$-induced carotid artery injury and the onset of rapid occlusive thrombosis is shown in Fig. 6. Once the mice entered the rapid occlusive thrombosis phase, a difference in the rate of occlusive thrombus formation was observed between the anti-$\beta_2$GPI/$\beta_2$GPI complex-injected and IgG-injected mice. At 1 min, the vessels of the complex-injected WT mice showed a slight decrease in relative blood volume, whereas IgG-injected WT or complex-injected *p38MAPK*$^{-/-}$ mice showed no clear change. After 5 min, the blood volume of the WT mouse carotid arteries injected with the complexes had nearly completely vanished. The interval

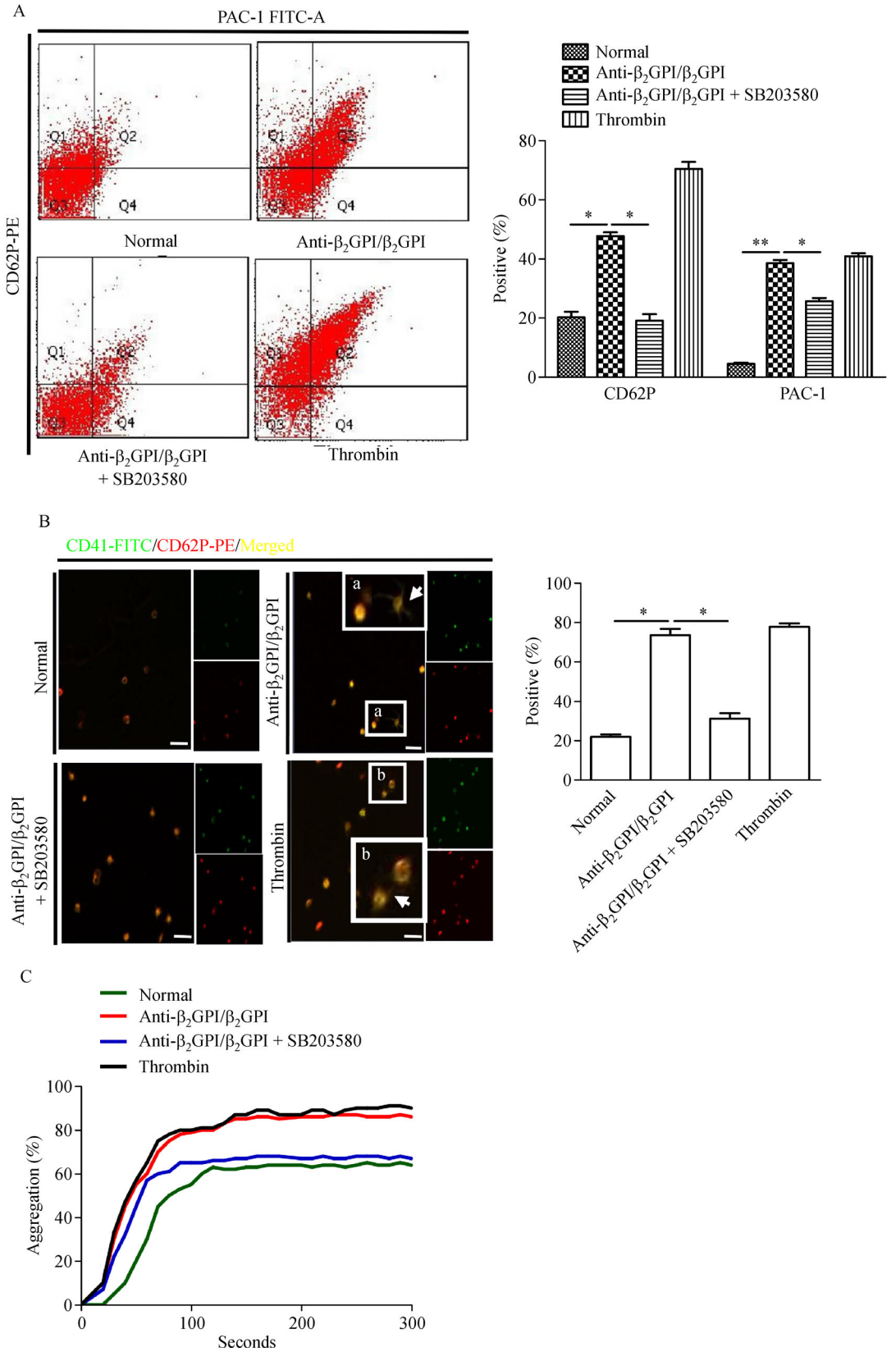


**Fig. 3** Anti-$\beta_2$GPI/$\beta_2$GPI complexes stimulated platelet activation, secretion, and aggregation via the p38 MAPK pathway. (A) P-selectin and GPIIb/IIIa were identified by anti-CD62P-PE antibody and anti-PAC-1-FITC antibody, respectively. The percentage of CD62P or PAC-1 positive platelets was then determined by flow cytometry. (B) Platelet-derived microparticles were stained with anti-CD62P-PE and anti-CD41-FITC antibodies and imaged via confocal microscopy. Numerous small projections were observed emanating from the platelet surface (marked by the white arrow). Scale bar: 10 μm. The percentage of CD62 and CD41-positive platelet microparticles was calculated by ImageJ. (C) Platelet aggregation was monitored using a turbidimetric aggregometer. Data are shown as the mean ± SEM and taken from a single experiment representative of three independent experiments. *$P < 0.05$, **$P < 0.001$.

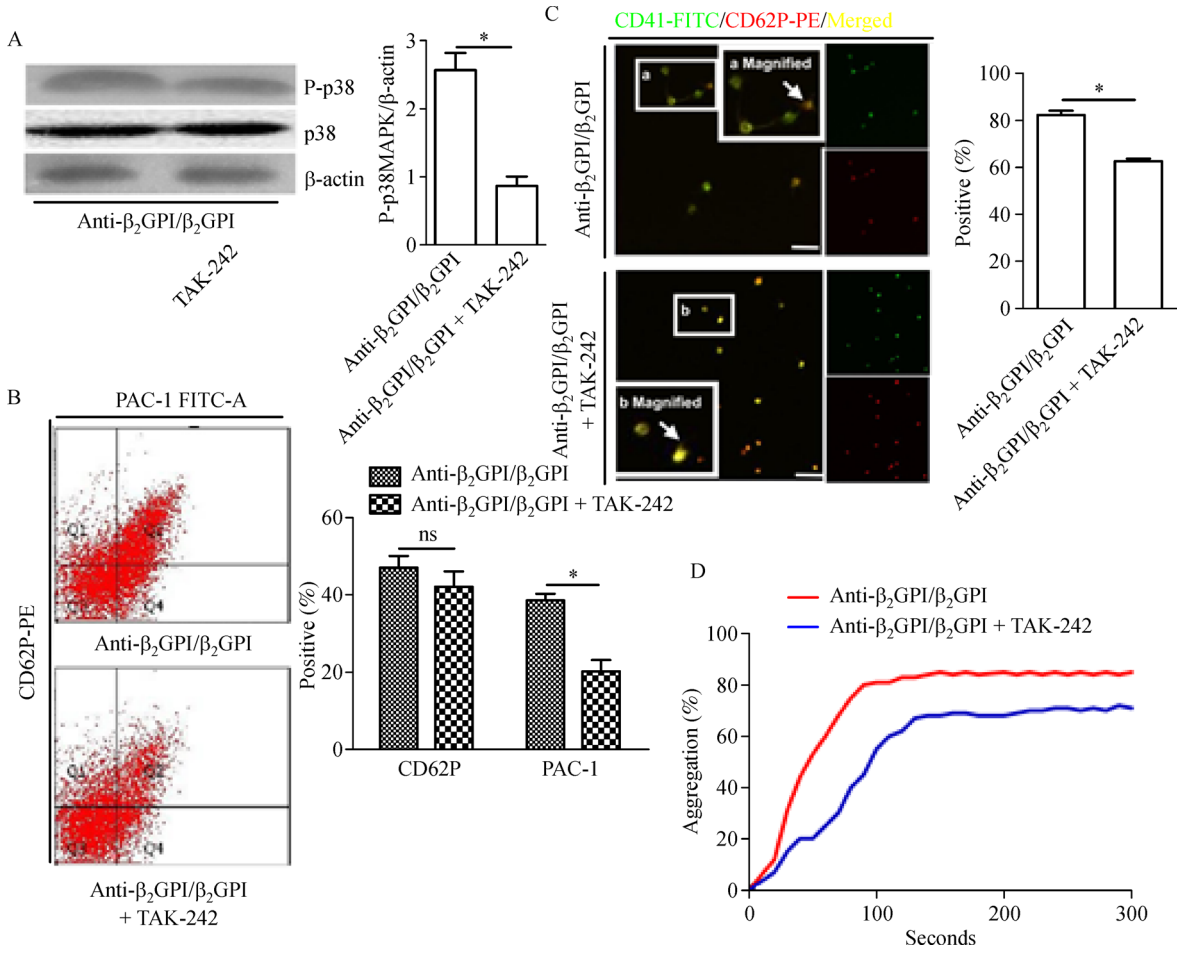


**Fig. 4** Anti-$\beta_2$GPI/$\beta_2$GPI complex-mediated p38MAPK activation and platelet activation can be decreased by inhibition of TLR4. Control platelets were stimulated with the anti-$\beta_2$GPI/$\beta_2$GPI complexes. Some samples were pretreated with TAK-242, a TLR4 inhibitor. (A) Phosphorylated-p38MAPK of platelet was detected by Western blot. (B) The percentage of CD62P or PAC-1 positive platelets was detected by flow cytometry. (C) Platelet-derived microparticles were imaged via confocal microscopy. Numerous small projections were observed emanating from the platelet surface (marked by the white arrow). Scale bar: 10 μm. The percentage of CD62 and CD41-positive platelet microparticles was calculated by ImageJ. (D) Platelet aggregation was monitored using a turbidimetric aggregometer. Data are shown as the mean ± SEM and taken from a single experiment representative of three independent experiments. *$P < 0.05$, ns = not significant.

from $FeCl_3$-induced arterial injury to occlusive thrombosis was significantly prolonged in *p38MAPK*$^{-/-}$ mice (Fig. 6A and 6B). From our histopathology analysis, we selected 5 min as the endpoint. At this time point, we removed the arteries and stained them with hematoxylin–eosin. We found that the thrombus size was greatly increased due to complex treatment but was decreased due to the *p38MAPK* knockout (Fig. 6C).

## Discussion

An unusual and compelling feature of anti-$\beta_2$GPI antibody-associated disease is that it predisposes patients to both arterial and venous thrombosis. In this work, we showed, for the first time, that platelets of anti-$\beta_2$GPI antibody-positive thrombotic patients feature extremely high levels of p38MAPK phosphorylation. We then found that this stimulation was partially dependent on addition of an exogenous source of $\beta_2$GPI, either as a purified protein or in serum. This observation is likely explained by the anti-$\beta_2$GPI-induced p38MAPK phosphorylation being impaired when the antigen support is lost.

Our *in vitro* work strongly supports a role for anti-$\beta_2$GPI/$\beta_2$GPI complexes in directly promoting p38MAPK phosphorylation and activation of platelets. The higher levels of p38MAPK phosphorylation observed may also be partially explained by a recent study demonstrating platelet adhesion stimulated by the complexes *in vitro* [7], which did not, however, measure p38MAPK phosphorylation in

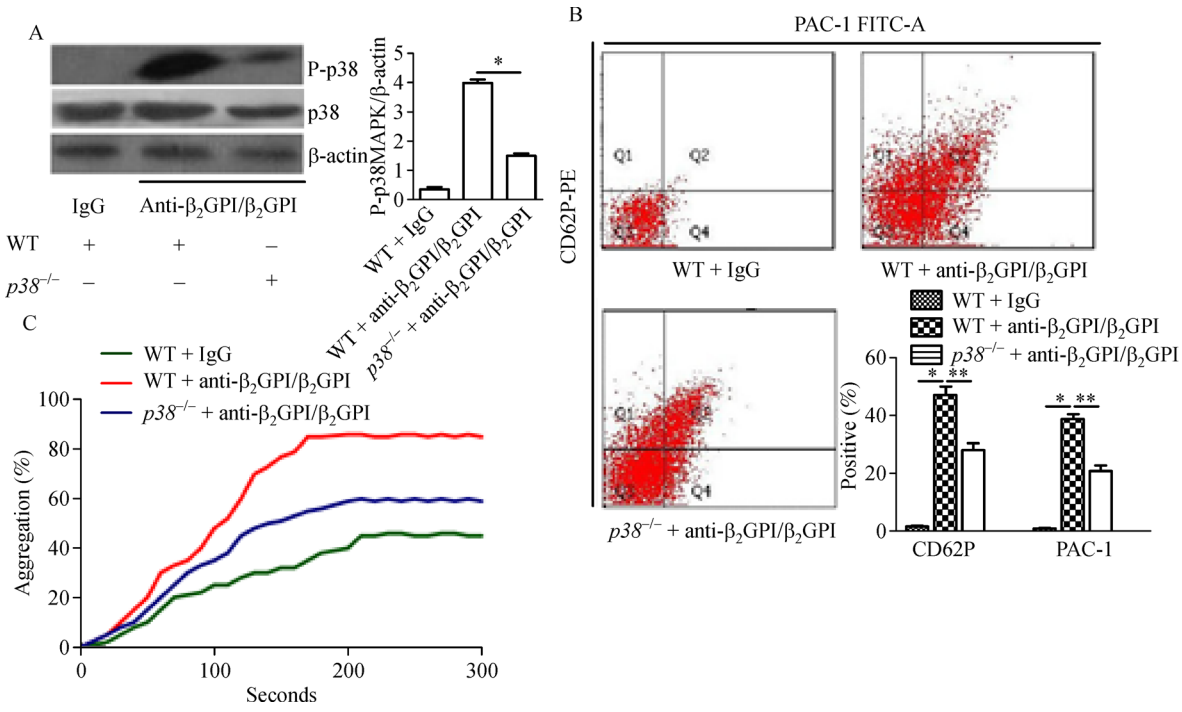


**Fig. 5** Anti-$\beta_2$GPI/$\beta_2$GPI complex-induced platelet activation *in vivo* is mediated by p38MAPK. WT mice were i.v. injected with human IgG or anti-$\beta_2$GPI/$\beta_2$GPI complexes. *p38MAPK*$^{-/-}$ mice were injected with anti-$\beta_2$GPI/$\beta_2$GPI complexes. (A) p38MAPK phosphorylation was tested in treated platelets by Western blot. (B) P-selectin and GPIIb/IIIa were identified by goat anti-mouse anti-CD62P-PE antibody and anti-PAC-1-FITC antibody. The anti-CD62P antibody and anti-PAC-1 antibody-positive platelets was detected by flow cytometry. (C) Platelet aggregation was monitored using a turbidimetric aggregometer. Data are shown as the mean ± SEM and taken from a single experiment representative of three independent experiments. *$P < 0.05$, **$P < 0.001$.

the platelets. Although anti-$\beta_2$GPI antibody could interact with cells via its Fc region, after the Fc region was blocked, the results of the p38MAPK phosphorylation induced by the complexes were not affected. This finding reveals that the anti-$\beta_2$GPI antibody may be lost in the preferential combination function of its Fc region once complexed to $\beta_2$GPI. Somewhat surprisingly, we found that, in antibody-positive nonthrombotic patients, the levels of anti-$\beta_2$GPI/$\beta_2$GPI complexes are much lower than those in thrombotic patients with anti-$\beta_2$GPI. This finding may be explained by some patients with lower levels of circulating anti-$\beta_2$GPI antibody not developing thrombosis in the short term since levels of anti-$\beta_2$GPI/$\beta_2$GPI complexes are not sufficient to induce p38MAPK phosphorylation completely, which then promotes thrombosis in some cases. This hypothesis is supported by the positive correlation between the anti-$\beta_2$GPI/$\beta_2$GPI complexes and p38MAPK phosphorylation.

Our previous study found that anti-$\beta_2$GPI/$\beta_2$GPI complex-induced platelet activation occurs via both GPIbα and apoER2′ [12]. In this study, we additionally found that anti-$\beta_2$GPI/$\beta_2$GPI complex-induced platelet activation is at least partially dependent on TLR4. Unlike the p38MAPK inhibitor, TAK-242 could not significantly reduce P-selectin expression by platelets. This effect may be explained by studies demonstrating that TLR4 activated by bacterial LPS does not induce P-selectin expression in wild-type platelets even when bacterial LPS is injected *in vivo* [14]. TLR4 clearly may not be the major receptor in the interaction between anti-$\beta_2$GPI/$\beta_2$GPI complexes and platelets, which could be explained by the high levels of P-selectin expression induced by the complexes.

*In vitro,* anti-$\beta_2$GPI/$\beta_2$GPI complexes stimulated platelet activation and promoted platelet aggregation and secretion, an effect that could be impaired by SB203580 treatment. We thus suggest that these data have implications for the prothrombotic diathesis inherent in anti-$\beta_2$GPI antibody-positive patients. To fully understand this pathway, we designed an animal investigation and confirmed that anti-$\beta_2$GPI antibody complexes with $\beta_2$GPI induce platelet activation and aggregation and promote p38MAPK phosphorylation in platelets from WT mice. The formation of anti-$\beta_2$GPI/$\beta_2$GPI complexes also appears to contribute toward a significant increase in the thrombus size of the carotid artery in an animal model of $FeCl_3$-induced vessel damage. A “two-hit hypothesis” has been proposed in which anti-$\beta_2$GPI/$\beta_2$GPI complexes (the first hit) induce a threshold cellular perturbation in platelets, such as p38MAPK phosphorylation, and another condition (the

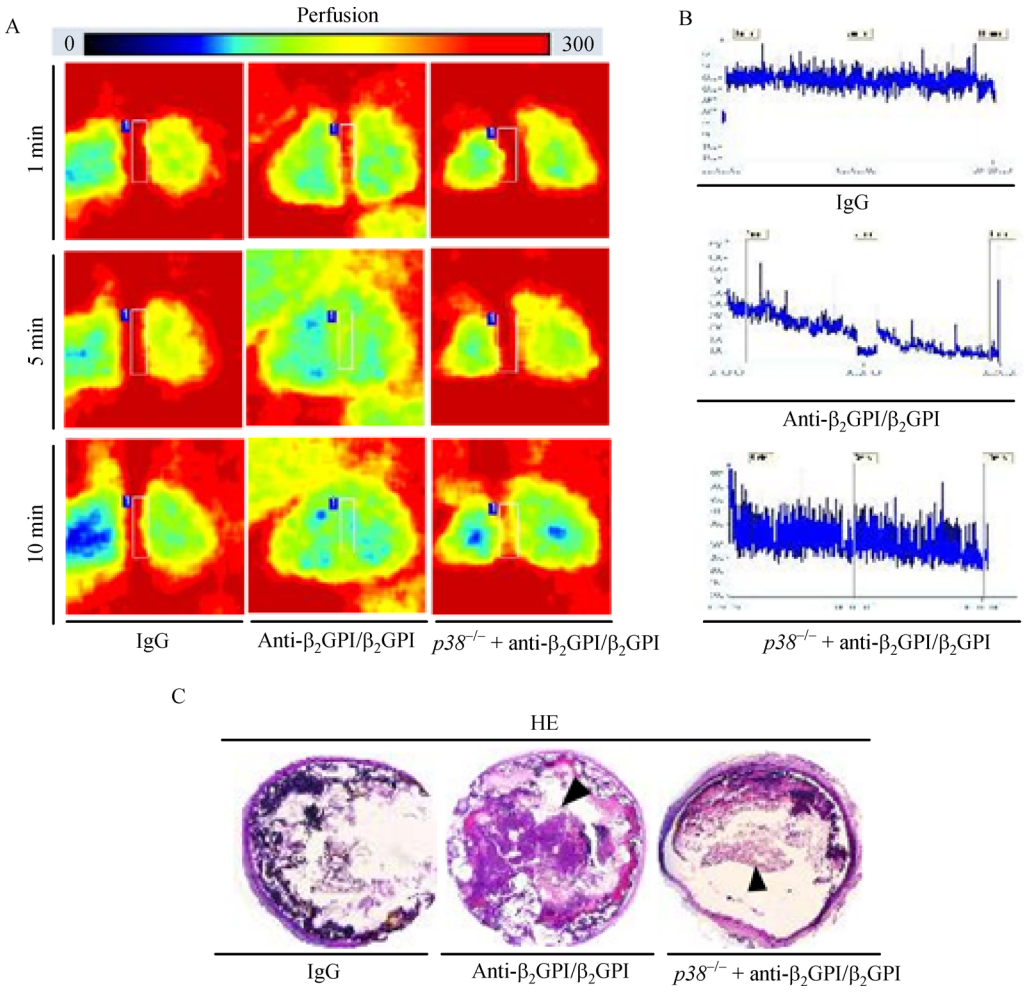


**Fig. 6** Inhibiting p38MAPK can prevent anti-$\beta_2$GPI/$\beta_2$GPI complex-induced thrombosis *in vivo*. The common carotid arteries were exposed, and filter paper saturated with 10% $FeCl_3$ was placed under the right vessel throughout the procedure. (A) Carotid artery thrombosis was measured at 1, 5, and 10 min via laser Doppler flowmetry. The rate of blood perfusion is shown as decreasing from red to blue. (B) Changes in carotid artery blood flow were measured over a duration of 10 min via laser Doppler flowmetry and identified by PIMsoft software. (C) The arteries were removed after treatment with 10% $FeCl_3$ for 5 min, and hematoxylin–eosin staining was used to measure the thrombus size. Representative photograph of a carotid artery cross-section showing the maximal thrombus size following anti-$\beta_2$GPI/$\beta_2$GPI complex treatment, which was reduced by *p38MAPK* knockout. Thirteen mice per group; data are from a single experiment representative of two independent experiments. ▲ shows the presence of a thrombus.

second hit) is required to trigger thrombus clot formation. In most prior studies of anti-$\beta_2$GPI antibody-associated thrombosis in rodents, as well as in our experiments, thrombus formation must be induced. The second hit may involve a proinflammatory stimulus (such as $FeCl_3$ in this study), since animals administered anti-$\beta_2$GPI/$\beta_2$GPI complexes show spontaneous thrombosis if they also receive LPS [15]. These observations support the hypothesis that inflammatory factors may play a dual role in anti-$\beta_2$GPI antibody-associated thrombosis pathogenesis by initially triggering the generation of cross-reactive anti-$\beta_2$GPI antibodies (first hit) due to considerable amino acid sequence homology between inflammation-associated plasma proteins (such as oxidized low density lipoprotein) and $\beta_2$GPI [16] and then by inducing an inflammatory response that provides the second hit.

p38MAPK has been a popular target for the design of anti-inflammatory drugs for well over a decade. The present finding showing that *p38MAPK*$^{-/-}$ mice exhibit decreased thrombus formation after anti-$\beta_2$GPI/$\beta_2$GPI complex treatment is an exciting discovery and indicates that the *p38MAPK* knockout can prevent platelet activation and aggregation in mice. Therefore, we speculate that modulation of the p38MAPK pathway could be of therapeutic benefit for downregulation of platelet activation and thrombosis induced by anti-$\beta_2$GPI/$\beta_2$GPI complexes.

Overall, this study clearly demonstrates that anti-$\beta_2$GPI/

$\beta_2$GPI complexes can induce platelet activation and contribute to thrombotic events via the p38MAPK pathway. p38MAPK may be a treatment strategy for anti-$\beta_2$GPI antibody-associated thrombotic events. The pathogenic mechanism of thrombosis in anti-$\beta_2$GPI-positive patients should be further assessed in experimental models with an eye toward more targeted approaches for the therapy of autoimmune-related thrombotic diseases.

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## Compliance with ethics guidelines

Wenjing Zhang, Caijun Zha, Xiumin Lu, Ruichun Jia, Fei Gao, Qi Sun, Meili Jin, and Yanhong Liu declare that they have no financial or other relationships that may lead to a conflict of interest for the present article. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Harbin Medical University Institutional Review Committee, China) and with the *Helsinki Declaration* of 1975, as revised in 2000 (5). Informed consent was obtained from all patients included in the study. Approval for the animal experimental studies was received from the Institutional Animal Care and Use Committees of Harbin Medical University.

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