cell line RAW264.7 (American Type Culture Collection number TIB-71) was maintained in an atmosphere of 5% $CO_2$ at 37°C in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated FCS containing penicillin and streptomycin. Human umbilical vein endothelial cells (HUVECs) (Kurabo, Tokyo, Japan) were maintained under 5% $CO_2$ at 37°C in HuMedia EB-2 (Kurabo).

### Procoagulant cell treatment

Prothrombin, monoclonal aPS/PT (231D) and mouse IgG were added to PBMCs or RAW264.7 cells at a concentration of 10 μg/ml and to HUVECs at a concentration of 15 μg/ml. The IgG aPS/PT fraction (500 μg/ml) or control IgG fraction (500 μg/ml) was added to the cells. Lipopolysaccharide (LPS) was used as positive control at a concentration of 100 ng/ml. The $Ca^{2+}$ concentration in each sample was adjusted to 2.5 mM, which was sufficient to facilitate the binding of prothrombin to phosphatidylserine. The cells were treated for 5 h for TF mRNA determination, for 15 min for p38 MAPK phosphorylation and for 12 h for clotting assay.

### Flow cytometry assay with IIF staining

To observe the binding of monoclonal antibody to the cell surface, a flow cytometry assay with IIF staining was performed. Mouse monoclonal antibodies and control IgG were added to RAW264.7 with or without prothrombin. Cells were washed and collected after 4 h of incubation. Diluted FITC-conjugated AffiniPure donkey anti-mouse IgG antibody (Sigma-Aldrich Co.) was added to the cell suspension and then analysed with a flow cytometry (FACS) analyser.

### RNA isolation and quantitative TaqMan real-time PCR

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and stored at −80°C until use.

Complementary DNA (cDNA) was generated using the SuperScript II first-strand cDNA pre-amplification system (Gibco BRL, Rockville, MD, USA) according to the random primer protocol provided by the manufacturer. The induction of mRNA was measured by real-time PCR using TaqMan Universal PCR Master Mix and gene-specific sets of Assay-on-Demand Gene Expression probes (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Messenger RNA samples were analysed in at least three similar real-time PCR procedures. Negative controls containing water instead of RNA were simultaneously run to rule out cross-contamination. Relative expression was quantified by the ΔΔCt method and normalized to GAPDH.

### Clotting assay

To evaluate the procoagulant activity of cells, the clotting time of PBMCs was measured using an automated STA-R coagulation analyzer (Diagnostica Stago, Asnière, France). After three washes in Tris-buffered saline (TBS) containing 0.05% Tween 20 (Sigma-Aldrich Co.) and 5 mM $CaCl_2$ (TBS-Tween-Ca), 2 ml of normal human plasma was added to 2 ml of cell fluid ($1 \times 10^6$ cells/ml). The reduction in the clotting time compared with the normal control sample was interpreted as increased coagulation function and was attributed to the expression of TF.

### Detection of intracellular signal protein phosphorylation

For parallel determination of the relative phosphorylation levels of intracellular signal proteins, particularly MAPKs and other serine/threonine kinases, an array assay was performed using the Human Proteome Profiler Array kit (R&D Systems, Minneapolis, MN, USA) following the standard procedure provided by the manufacturer. Briefly, concentrated PBMC lysates obtained from normal healthy controls were adjusted according to the manufacturer's instructions following exposure to the stimulators [231D (10 μg/ml) with and without prothrombin (10 μg/ml)] for 15 min. The lysates were added to the array and exposed to X-rays for 5 min.

### Quantitative analysis of serine-threonine kinase phosphorylation by cellular activation ELISA

Quantitative analysis of intracellular signal phosphorylation in RAW264.7 mouse monocytes was performed using a Cellular Activation of Signaling ELISA (CASE) kit (SABiosciences Corporation, Frederick, MD, USA) following the standard method provided by the manufacturer. The phosphorylation of p38 MAPK, c-Jun N-terminal kinase (JNK), extracellular regulated kinase (ERK1/2) and Akt (protein kinase B) was carried out as follows. Briefly, experimentally treated cells were seeded in 96-well plates and fixed with paraformaldehyde. Two primary antibodies, one that recognizes phosphorylated serine-threonine kinases and another that recognizes serine-threonine kinases regardless of phosphorylation were used to detect the relative amount of phosphorylated serine-threonine kinases, which was assayed by measuring the optical density (OD) on an ELISA plate reader. The OD was measured at 450 nm and normalized to the cell number ($OD_{540}$). Then the OD ratio ($OD_{450}$:$OD_{540}$) of phospho-serine-threonine kinase-specific antibody (OD phospho-kinases) was normalized to the pan-serine-threonine kinase-specific antibody OD ratio (OD pan-kinases) under the same experimental conditions, indicating the relative extent of serine-threonine kinase phosphorylation (OD phospho-kinases/OD pan-kinases). Finally, to determine the relative extent of target protein phosphorylation, the OD phospho-kinases/OD pan-kinases ratio of each sample was compared with unstimulated samples to calculate the relative amount of serine-threonine kinase phosphorylation.

### RNA interference

RNA interference was carried out with Accell small interfering RNA (siRNA; Dharmacon, Lafayette, CO, USA), predesigned pools of four oligonucleotides, using the Accell siRNA delivery protocol following the manufacturer's

instructions. Briefly, 8 h after plating, PBMC from healthy controls ($5 \times 10^5$ cells/well) were transfected with 1 μM p38 MAPK-α (MAPK 14) Accell siRNA or Accell non-targeting siRNA in 100 μl Accell siRNA delivery media (Dharmacon). Cells were incubated at 37°C 72 h before assessment of RNAi knockdown effect.

### Proteins

Fatty acid-free BSA was obtained from Sigma-Aldrich. LPS was removed from the antibody preparation by using DetoxiGel (Pierce, Rockford, IL, USA), and its absence was confirmed using the Limulus amebocyte lysate assay (Limulus ES-II Single Test Wako; Wako, Osaka, Japan). Human prothrombin was obtained from Enzyme Research (South Bend, IN, USA).

### Statistical analysis

Means of the various treated and control groups were compared by Student's unpaired *t*-test. SPSS II for Windows (SPSS Japan Inc., Tokyo, Japan) was used for all calculations.

## Results

### Upregulation of TF mRNA expression in PBMCs and RAW264.7 cells treated with IgG from APS patients' plasma and monoclonal aPS/PT

Immunoglobulin G isolated from APS patients' plasma and LPS significantly increased the expression of TF mRNA in PBMCs. In contrast, IgG from healthy controls did not increase the expression of TF mRNA in PBMCs (Fig. 1A).

231D in the presence of prothrombin significantly increased the expression of TF mRNA in PBMCs and in RAW264.7 cells. However, 231D in the absence of prothrombin or control IgG with prothrombin did not increase TF mRNA expression (Fig. 1B and C).

### Procoagulant activity of PBMCs treated with aPS/PT

TF function in aPS/PT-treated cells, measured by procoagulant activity, was analysed using a clotting assay. The clotting time of the cell fluid from PBMCs treated with IgG isolated from APS patients' plasma in the presence of prothrombin was significantly reduced. In contrast, the coagulation time of cell fluid treated with APS patients' IgG alone or IgG from healthy controls with prothrombin was not reduced (Fig. 2A). In addition, the clotting time of the cell fluid from PBMCs treated with 231D in the presence of prothrombin was significantly reduced. The coagulation time of cell fluid treated with 231D alone or with control IgG and prothrombin was not reduced (Fig. 2B).

### Monoclonal aPS/PT binding to the cell surface of RAW264.7 cells was detected by a flow cytometric assay with IIF staining

RAW264.7 were treated with monoclonal aPTs (51A6 and 231D) or control mouse IgG at 37°C under 5% $CO_2$ for 4 h. In the presence of prothrombin, 75.9% of 231D-treated cells bound to antibody, while only 41.4% of 51A6-treated cells and 0.5% of control IgG-treated cells bound to antibody. In the absence of prothrombin, cells treated with antibodies showed almost no binding to the 231D, 51A6 and control IgG antibodies (3.8%, 0.1% and 0.3%, respectively) (Fig. 3).

### Intracellular signal protein phosphorylation in PBMCs treated with monoclonal aPS/PT

Results of the array assay showed phosphorylation of p38 (p38α) in PBMCs treated with 231D in the presence of prothrombin. However, no p38 phosphorylation was detected in cells treated with 231D in the absence of prothrombin. Phosphorylation of other serine/threonine kinases or other MAPK family proteins was also not detected, therefore p38 was presumed to be the major signal protein involved in monocyte activation by aPS/PT.

### Quantitative analysis of intracellular signal phosphorylation in RAW264.7 cells treated with monoclonal aPS/PT

Based on the results of the array assay, serine-threonine kinases including p38 phosphorylation was quantitatively analysed using an ELISA CASE kit. In the presence of prothrombin, 231D significantly increased the relative amount of p38 phosphorylation compared with the untreated control up to 1.7-fold. There was no increase in the amount of relative p38 phosphorylation with 231D in the absence of prothrombin, or with control mouse IgG plus prothrombin (Fig. 4A). The relative amount of phosphorylation in other serine-threonine kinases such as JNK, ERK1/2 and Akt were not detected (Fig. 4B–D).

### Effect of p38 MAPK inhibitor on PBMCs TF expression induced by monoclonal aPS/PT treatment

To elucidate the role of p38 MAPK in TF mRNA expression, we investigated the effect of a p38 MAPK inhibitor on cells treated with monoclonal aPS/PT. The p38-specific inhibitor SB203580 significantly reduced TF mRNA overexpression in 231D-treated PBMCs (Fig. 5A) and RAW264.7 cells compared with the untreated control (Fig. 5B). However, its inactive analogue SB202474 did not affect TF mRNA expression. Addition of SB203580 to 231D-treated cells decreased TF mRNA expression 80–90%.

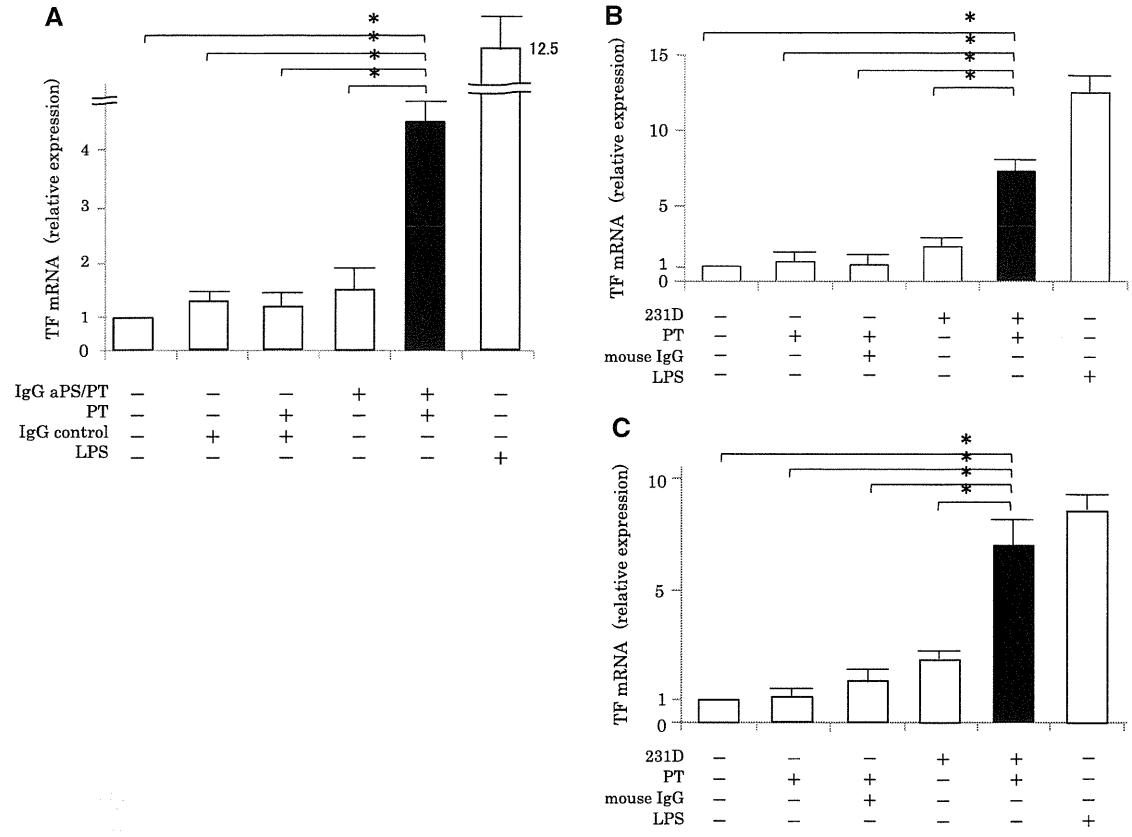
### Effect of siRNA reagents on PBMC TF expression induced by monoclonal aPS/PT treatment

The effect of p38 siRNA on PBMC TF mRNA expression induced by 231D treatment was investigated as indicated. The expression of TF mRNA on 231D-treated PBMCs was significantly offset by pre-treatment of p38 siRNA. In contrast, pre-treatment of control siRNA did not affect TF mRNA expression on 231D-treated PBMCs (Fig. 6).

### Upregulation of TF mRNA expression and adhesion molecules in HUVECs induced by monoclonal aPS/PT

The expression of TF mRNA was significantly upregulated in HUVECs treated with 231D in the presence of

FIG. 1 Upregulation of TF mRNA expression by aPS/PT.




RAW264.7 cells or PBMCs obtained from normal healthy controls were exposed to the substances described below for 5 h. IgG extracted from APS patients positive for aPS/PT (IgG aPS/PT) or IgG extracted from healthy controls (IgG control) was added at 500 μg/ml. Prothrombin (PT), 231D and mouse control IgG (mouse IgG) were added at a concentration of 10 μg/ml and LPS was added at a concentration of 100 mg/ml. *$P < 0.005$. Vertical axes represent the relative expression levels of TF mRNA determined by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. (**A**) The relative TF mRNA expression levels in PBMCs treated with an IgG fraction from aPS/PT-positive patients or an IgG fraction from healthy controls. (**B**) Relative TF mRNA expression levels in PBMCs treated with 231D or mouse control IgG. (**C**) The relative TF mRNA expression levels in RAW264.7 cells treated with 231D or mouse control IgG were measured.

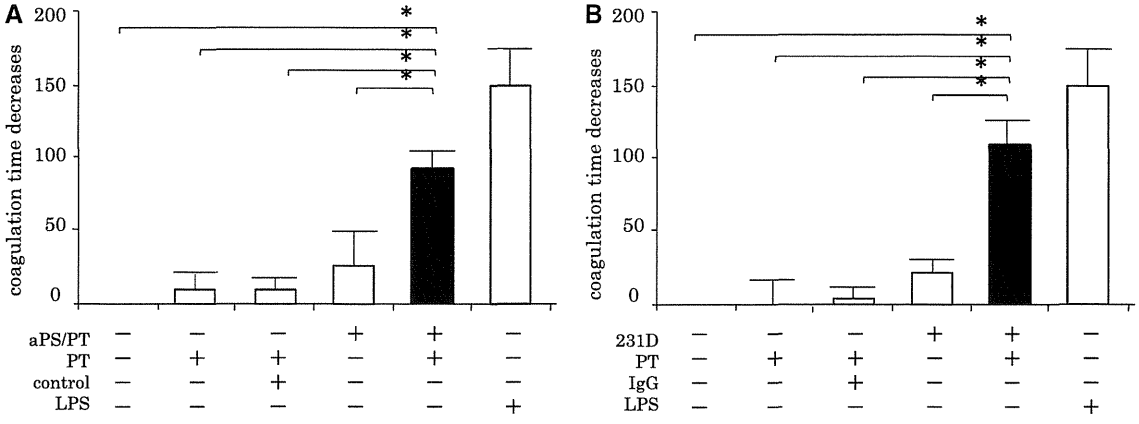
prothrombin (PT + 231D *vs* baseline, 231D alone and PT + control IgG: 2.5 ± 0.7 *vs* 1, 1.2 ± 0.3 and 1.31 ± 0.24; all, $P < 0.005$). The p38-specific inhibitor SB203580 significantly reduced TF mRNA overexpression in 231D-treated HUVECs [SB203580 (+) *vs* SB203580 (−): 1.4 ± 0.2 *vs* 2.5 ± 0.7; $P < 0.005$]; however, its inactive analogue SB202474 did not affect TF mRNA overexpression.

The expression levels of vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PCAM-1) and endothelin-1 mRNA were significantly upregulated 2- to 3-fold in HUVECs treated with 231D in the presence of prothrombin. However, in the absence of prothrombin, 231D did not affect the expression of these adhesion molecules (PT + 231D *vs* baseline, 231D alone and PT + control IgG; VCAM-1: 2.1 ± 0.6 *vs* 1, 1.4 ± 0.1 and 1.1 ± 0.2, PCAM-1: 2.8 ± 0.3 *vs* 1, 1.3 ± 0.0 and 1.8 ± 0.3, selectin: 2.0 ± 0.4 *vs* 1, 1.3 ± 0.2 and 1.1 ± 0.4; all, $P < 0.01$).

## Discussion

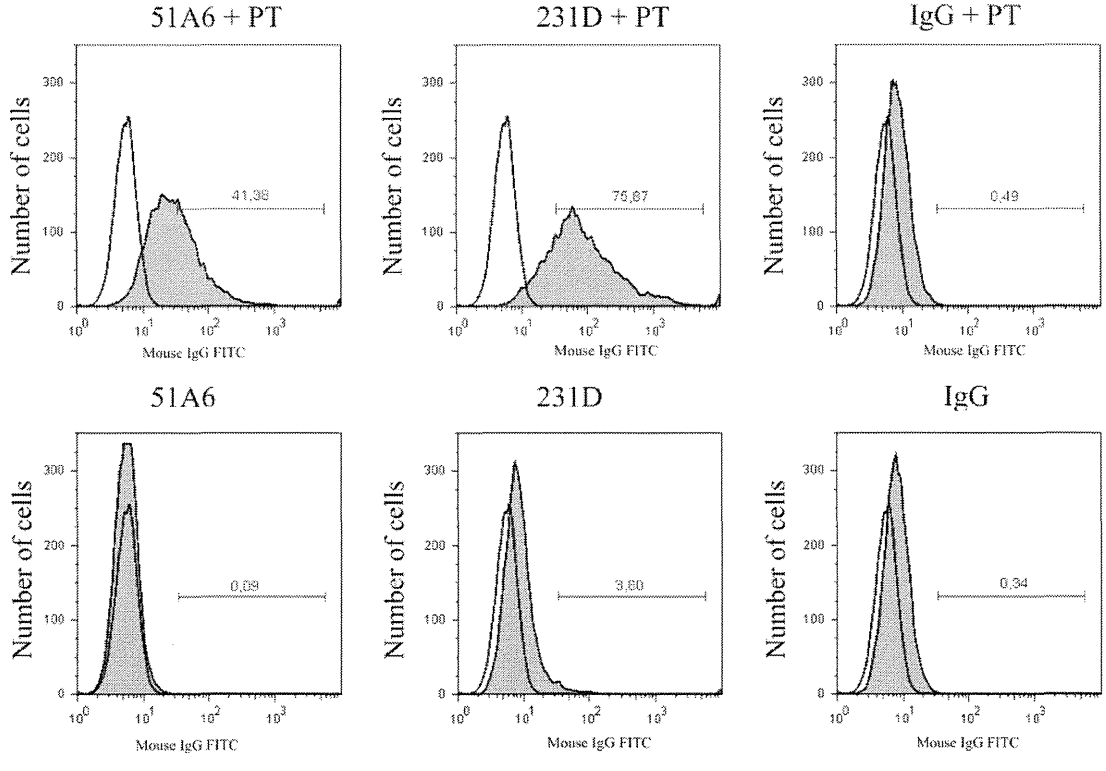
In this study we showed that IgG fractions and monoclonal aPS/PT induced TF in procoagulant cells. Monoclonal aPS/PT bound to monocytes using prothrombin as a cofactor for binding. Further, we demonstrated that treatment by aPS/PT induces the phosphorylation of p38 MAPK in these procoagulant cells. The coagulation process *in vivo* is complicated and various cells or molecules other than monocytes or endothelial cells are involved. Obviously there are certain limitations in discussing aPS/PT-induced thrombosis from the current results. However, our results showed that TF, the key protein in the coagulation pathway, is overproduced by its main sources in the circulation, monocytes and endothelial cells. Upregulation of blood-borne TF indicates increased procoagulant activity that is considered one of the most important characteristics of aPL-induced thrombosis.

Fig. 2 Procoagulant activity of cells treated with aPS/PT.



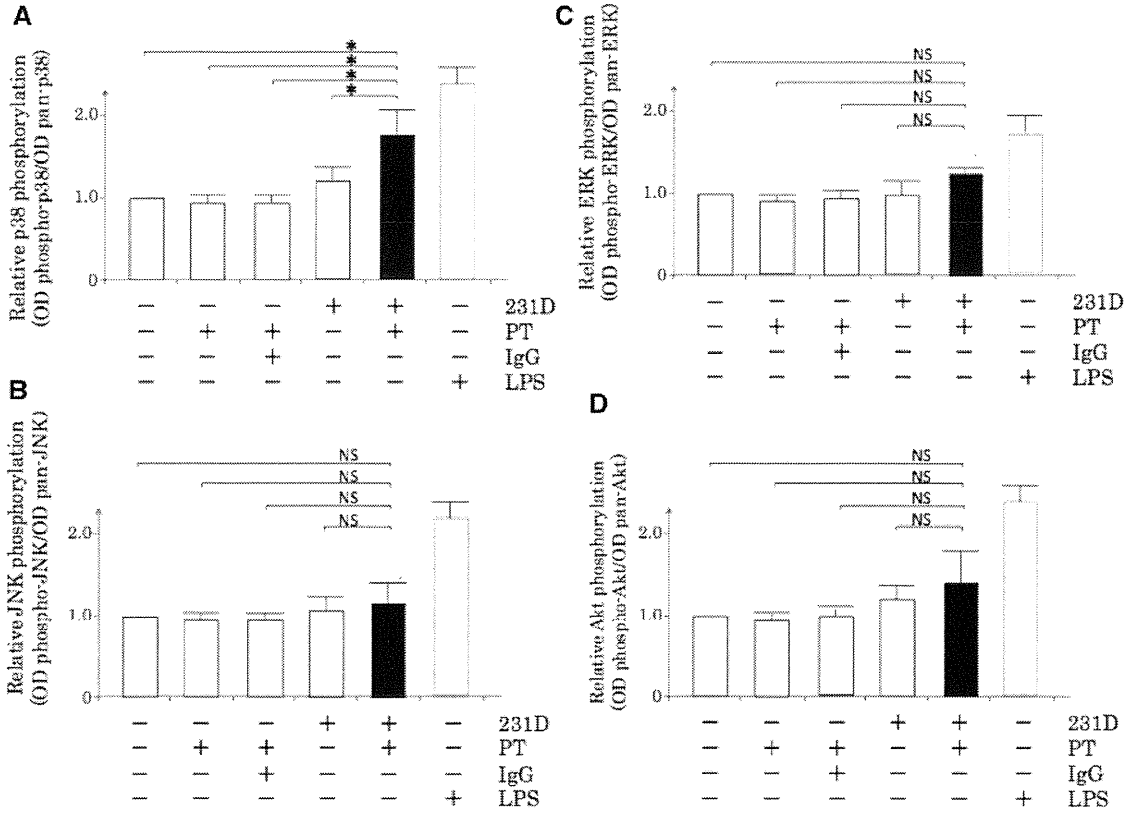

PBMCs obtained from normal healthy donors were exposed to the substances described below for 5 h. The cells were washed and then added to normal healthy plasma, and coagulation time was measured. The reduction in coagulation time was calculated by subtracting the coagulation time of each treated sample from that of unstimulated cells. The bars represent the mean ± S.E. of three independent experiments. *$P < 0.005$. (**A**) PBMCs treated with IgG fractions from patients positive for aPS/PT (aPS/PT) or IgG fraction from healthy controls (control) (500 μg/ml) in the presence or absence of prothrombin (PT). (**B**) PBMCs treated with 231D or mouse control IgG (IgG) (10 μg/ml).

Fig. 3 Binding of aPS/PT to the surface of RAW264.7 cells.



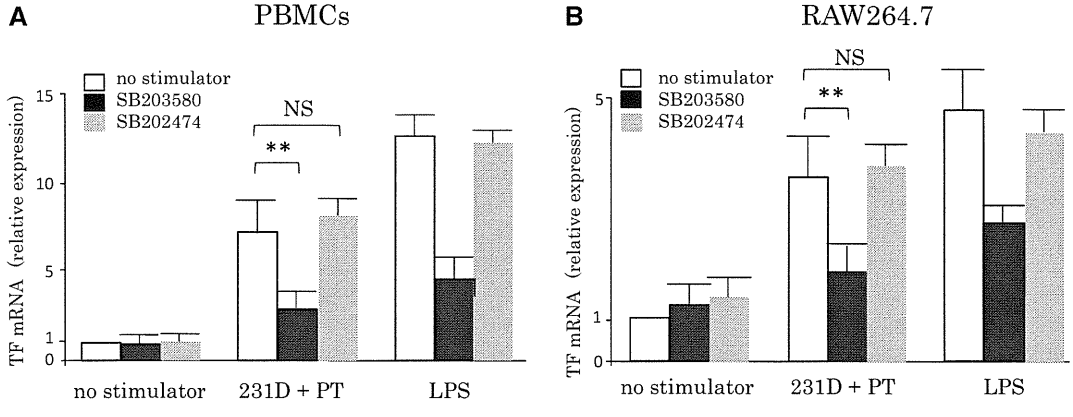

Murine monoclonal anti-prothrombin antibodies (51A6, 231D) and control murine IgG (IgG) were added to RAW264.7 cells at a concentration of 7.5 μg/ml with or without prothrombin (PT) (10 μg/ml), and then incubated for 4 h. After incubation, FITC-conjugated anti-mouse IgG antibody was added to the cell suspension and then analysed with a FACS analyzer. The vertical axes represent the number of cells and the horizontal axes represent the FITC fluorescence intensity.

FIG. 4 Quantitative analysis of serine-threonine kinase phosphorylation in aPS/PT-treated cells.



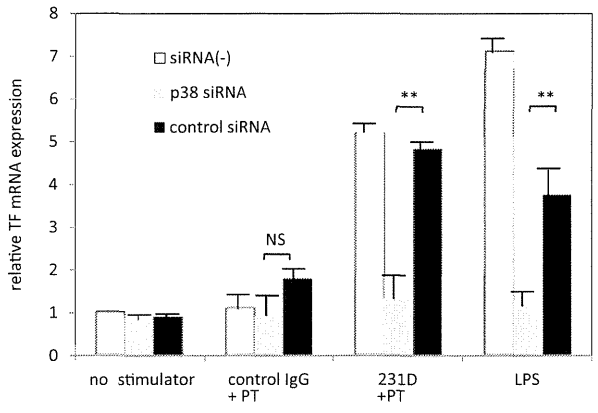

RAW264.7 cells were exposed to the substances described below for 15 min. Prothrombin (PT), 231D and mouse control IgG (IgG) were added at a concentration of 10 μg/ml and LPS was added at a concentration of 100 ng/ml. The relative OD ratio of each sample was measured and calculated as described in the Materials and methods section. *$P < 0.005$, NS: not statistically significant.

FIG. 5 p38 MAPK inhibition assay in aPS/PT-treated cells.




PBMCs and the mouse monocyte cell line, RAW264.7, were exposed to stimulators for 5 h. Prothrombin (PT) and 231D were added at a concentration of 10 μg/ml and LPS was added at 100 ng/ml. Cells were pretreated with the p38-specific inhibitor SB203580 (1 μM) or its inactive analogue SB202474 (1 μM) for 30 min followed by treatment with stimulators. Vertical axes represent the relative expression levels of TF mRNA detected by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. **$P < 0.001$, NS: not statistically significant. (**A**) The relative TF mRNA expression levels in PBMCs. (**B**) The relative TF mRNA expression levels in RAW264.7 cells.

FIG. 6 RNA interference of p38 MAPK in aPS/PT-treated cells.




PBMCs were pretreated for 72 h with siRNA of p38 MAPK or the control and subsequently exposed to stimulators for 5 h. Prothrombin (PT) and 231D were added at a concentration of 10 μg/ml and LPS was added at 100 ng/ml. The vertical axis represents the relative expression level of TF mRNA detected by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. $^{**}P < 0.001$, NS: not statistically significant.

231D and 51A6 significantly bound to the membranes of monocytes in the presence of prothrombin. The binding of 231D to the cell surface was clearly stronger than that of 51A6. This observation was similar to our previous report that 231D had stronger binding to the PS/PT complex than 51A6 [13], suggesting that the monoclonal antibodies bind to prothrombin complexed with phosphatidylserine on the cell surface.

Our data suggest that TF production induced by aPS/PT in procoagulant cells is mainly induced via activation of the p38 MAPK pathway, which is similar to past reports showing that p38 MAPK was the main pathway of aCL/$\beta_2$GPI-induced cell activation. It is interesting that antibodies recognizing different proteins seem at least partially to utilize a common signalling pathway. Our findings are in agreement with the clinical observation that the manifestations of APS do not differ in patients with different antibody profiles.

Protein kinases are key regulators of cellular signalling, inflammation, cell differentiation and cell death. Thus they have been attractive targets for the treatment of neoplasms and inflammatory diseases [25–27].

p38 MAPK belongs to the MAPK signal protein family and is strongly activated by environmental stress or inflammatory cytokines such as TNF-α, IL-1β and IL-18 [28–30]. Consequently p38 MAPK activation is considered critical for physiological immune responses, and p38 MAPK dysfunction is related to the pathology of autoimmune diseases other than APS [31–33].

In the present study, phosphorylation of signal proteins, such as those in the MAPK protein family and serine/threonine kinases, was screened in aPS/PT-treated cells using a proteome array and major signals were quantitatively measured by ELISA tests. No proteins other than p38 MAPK were found to be phosphorylated. Further, specific p38 MAPK inhibitors or knockdown of p38 MAPK mRNA effectively inhibited procoagulant cell activation. Therefore p38 MAPK is suggested as a major signal protein for the activation of aPS/PT-induced procoagulant cells. Although a previous study showed that ERK activation was observed in cells treated with IgG fractions from APS patients [22], an ERK inhibitor did not abolish TF expression in procoagulant cells, suggesting that ERK does not play a major role in cell activation. We also did not detect the ERK phosphorylation in our aPS/PT-treated cells.

The two major aPLs, aCL/$\beta_2$GPI and aPS/PT, are suggested to activate procoagulant cells primarily through p38 MAPK phosphorylation, therefore inhibition of p38 MAPK appears to be a promising modality for the treatment of APS. Since p38 MAPK contributes to various cell activities, its non-specific inhibition might result in severe complications. In fact, clinical trials of p38 inhibitors for a variety of diseases have been carried out; however, most of the trials encountered several complications and were unsuccessful [34]. A more realistic and practical strategy would be to target a more specific molecule involved in the activation of aPL-induced procoagulant cells.

Some reports have demonstrated possible receptors for aCL/$\beta_2$GPI-induced cell activation on procoagulant cells. Annexin A2 is a receptor for tissue plasminogen activator and plasminogen that is found on the surface of ECs and monocytes, and on the brush-border membrane of placental syncytiotrophoblasts, all of which are recognized targets of pathogenic aPLs [35, 36]. Annexin A2 interacts with the $\beta_2$GPI-aCL/$\beta_2$GPI complex on EC and monocyte surfaces, mediating cell activation [37–39]. The involvement of annexin A2 in aPL-mediated pathogenic effects has been reported *in vitro* and *in vivo* [40, 41]. However, it is still not clear whether such a receptor is actually involved in cell activation because annexin A2 is not a transmembrane protein. Further, it has been proposed that activation of the signalling responses required another transmembrane adaptor protein(s) that associates with annexin A2 on the EC surface [42].

The Toll-like receptor (TLR) family, in particular, TLR-2 and TLR-4 [43–45], may also play a role in the interaction of the $\beta_2$GPI-aCL/$\beta_2$GPI complex [42]. Adhered $\beta_2$GPI interacts with TLR-4 and aCL/$\beta_2$GPI cross-links $\beta_2$GPI and the TLR-4 complex, eventually triggering the signalling cascade activation. Moreover, TLR-4 is the putative adaptor protein for annexin A2 [38].

Further investigations have shown that megalin/gp330 [46] and apolipoprotein E receptor 2′ [47–49] are putative receptors for aCL/$\beta_2$GPI. Recently we identified the gelsolin/integrin α5β1 complex as a novel receptor of aCL/$\beta_2$GPI [50].

In contrast to the intensive investigation of aCL/$\beta_2$GPI thrombogenicity, no data are available on the mechanism of aPS/PT-dependent procoagulant cell activation. It is not yet known if aCL/$\beta_2$GPI and aPS/PT have a common

cell surface receptor and upstream signals of p38 MAPK. However, we believe that our data are the first to show the critical pathway of the procoagulant state related to antibodies against prothrombin.

There are several reports showing the correlation between aPS/PT and APS-related pregnancy morbidity [51–53] that are subject to further investigation to clarify the molecular mechanism of the manifestation. Identification and comparison of the receptors for aPS/PT and aCL/$\beta_2$GPI will help elucidate the pathogenicity of aPLs and the mechanisms of APS pathology.

**Rheumatology key messages**

- Phosphatidylserine-dependent aPT induced TF expressions on procoagulant cells *in vitro*.
- Similar to aCL, phosphatidylserine-dependent aPT induced cell activation via the p38 MAPK pathway.
- Cell activation via the p38 MAPK pathway may partially explain the pathogenesis of APS thrombosis.

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PAPER

# The involvement of CD36 in monocyte activation by antiphospholipid antibodies

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**Background:** CD36, known as a scavenger receptor, is a transmembrane glycoprotein expressed on monocytes, platelets and endothelial cells, recognizes multiple ligands, including phosphatidylserine, and regulates atherogenesis and thrombosis. The objective of this study is to investigate the possible involvement of CD36 in the pathophysiology of thrombosis in patients with antiphospholipid syndrome (APS). **Methods:** First, rs3765187, a missense mutation linked to CD36 deficiency, was investigated by TaqMan polymerase chain reaction (PCR) genotyping method in 819 Japanese, including 132 patients with APS, 265 with systemic lupus erythematosus (SLE) in the absence of APS, and 422 healthy subjects. Then, the involvement of CD36 in antiphospholipid antibody (aPL)-induced tissue factor (TF) expression was examined using CD36-null mice or anti-CD36. Purified IgG from patients with APS and a monoclonal phosphatidylserine-dependent antiprothrombin antibody were used in these experiments. TF expression was tested by real-time PCR and flow cytometry. **Results:** Minor allele carrier of rs3765187 was less frequent in patients with APS (3.8% $p=0.032$), but not in patients with SLE in the absence of APS (7.9% $p=0.32$), compared with healthy subjects (10.2%). The aPL-induced TF expression was significantly suppressed on peritoneal macrophages from CD36-null mice compared to wild type and significantly inhibited by anti-CD36 on human monocytes. **Conclusions:** The gene mutation linked to CD36 deficiency was less frequent in patients with APS. The deficient or suppressed CD36 function significantly reduced aPL-induced TF expression in vitro. Taken together, in a susceptible background CD36 scavenger receptor function may be involved in the thrombotic pathophysiology in patients with APS. *Lupus* (2013) **22,** 761–771.

**Key words:** Antiphospholipid syndrome; lupus anticoagulant; thrombosis; scavenger receptor

## Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder in which vascular thrombosis or pregnant morbidity occurs in patients having persistent laboratory evidence of antiphospholipid antibodies (aPL). It correlates with a poor prognosis or impaired activity of daily living for a high relapse rate of thrombosis.[1]

Pathogenic aPL contains β2 glycoprotein I-dependent anticardiolipin antibodies (aCL/β2GPI) and phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT).[2,3] Both antibodies recognize epitopes on the phospholipid-binding proteins (β2GPI or prothrombin) interacting with negatively charged phospholipids, such as cardiolipin and phosphatidylserine. It is commonly believed that these epitopes express only when the phospholipid-binding proteins bind with anionic phospholipids.[4] In the 1990s, the function of phospholipid-binding protein was extensively studied based on the hypothesis that the interaction of aPL with their antigens impairs their anti- or procoagulant activities. However, the activation of procoagulant cells (monocyte, endothelial cell and platelet) through the binding of phospholipid-binding protein and aPL has been the focus of investigations during the last decade.[1]

Tissue factor (TF) upregulation has been advocated as one of the most important mechanisms in the pathogenesis of APS. Monocytes and endothelial cells treated with aPL demonstrate upregulation of TF expression and function, which is accompanied by an increase in interleukin (IL)-6

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or other proinflammatory substances.[5,6] Enhanced TF expression has been observed in healthy monocytes incubated with polyclonal or monoclonal aPL.[6] The activation of nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) has been recognized as participating in this process as intracellular signaling pathways.[7,8] However, cell surface receptor participation in this process is still controversial, and a number of candidates have been reported, including annexin A2, apolipoprotein E receptor 2', glycoprotein Ibα, low-density lipoprotein (LDL) receptor-related protein, megalin, toll-like receptor (TLR)2, TLR4, very-low-density lipoprotein, P-selectin glycoprotein ligand-1 and integrin α5β1.[9–16]

CD36, known as a member of class B scavenger receptors, is an 88-kDa transmembrane glycoprotein expressed on monocytes, macrophages, platelets and capillary endothelial cells, residing in lipid raft domains.[17,18] CD36 recognizes multiple ligands, including anionic phospholipids, oxidized LDL, long-chain fatty acid, collagen, thrombospondin-1 and malaria-infected erythrocytes and plays a role as a mediator of multiple functions including inflammation, atherogenesis and thrombosis through the activation of p38 and JNK MAPK and NF-κB.[18,19] Human CD36 deficiency was first described in 1989 in subjects refractory to human leukocyte antigen (HLA)-matched platelet transfusions[20] and is found in 4% to 10% of Asian or African populations.[21–23] It is divided into two subgroups: In type I deficiency, neither monocyte nor platelet expresses CD36, while in type II deficiency monocyte CD36 is expressed in the absence of platelet CD36.[24] There are three major polymorphisms on the exons of CD36 gene: a missense mutation linked to human CD36 deficiency in the Japanese population (rs3765187, C478T, Pro90Ser),[21] a nonsense mutation linked to human CD36 deficiency in the African population (rs3211938, T1264G),[25] and a mutation on the 5' untranslated region (rs1049654) whose clinical significance is unknown. Some clinical phenotypes of CD36 deficiency have been reported, including hypertrophic cardiomyopathy, hypertension and dyslipidemia.[26–28] The phenotypes of CD36 knock-out (KO) mice have been reported to be protective for atherosclerosis, thrombosis and inflammation, but susceptible to infection.[19,29–31]

Considering the distribution, ligands and function of CD36, we hypothesized that CD36 is involved in the pathogenesis of APS as one of the surface receptors on procoagulant cells, and thus performed a genetic and molecular-biologic investigation.

## Material and methods

### *Patients*

A total of 819 Japanese subjects, including 132 patients with APS, 265 with systemic lupus erythematosus (SLE) in the absence of APS and 422 healthy subjects, were enrolled. All the patients fulfilled the Sydney-revised Sapporo criteria of APS[32] and/or the American College of Rheumatology classification criteria of SLE.[33] Profiles of the patients with APS are shown in Table 1. This study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the local ethics committee and informed consent was obtained from each study subject before enrollment.

### *Determination of aPL*

Two clotting tests were performed for lupus anticoagulant (LA) determination, using a semiautomated hemostasis analyzer (STart 4; Diagnostica Stago, Asnières-sur-Seine, France) according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis.[34] For measurement of the activated partial thromboplastin time (APTT), a sensitive reagent with a low phospholipid concentration (test PTT-LA; Diagnostica Stago) was used for screening, and the results were confirmed with the use of a StaClot LA kit

**Table 1** Profiles of patients with APS

| *Total number* | | *132* |
|---|---|---|
| Age (year) | | 42 (15–74) |
| Female | | 111 |
| Primary APS | | 61 |
| Complicated SLE | | 71 |
| Clinical manifestations (overlapping) | | |
| | Arterial thrombosis | 83 |
| | Venous thrombosis | 43 |
| | Pregnant morbidity | 38 |
| Autoantibodies (overlapping) | | |
| | LA | 107 |
| | aCL | 73 |
| | aβ2GPI | 66 |
| | aPS/PT | 92 |

APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus; LA: lupus anticoagulant; aCL: anticardiolipin antibodies; aβ2GPI: anti-β2 glycoprotein I antibodies; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies.

(Diagnostica Stago). The dilute Russell's viper venom time (dRVVT) was screened for and confirmed by use of a Gradipore LAC test (Gradipore, Sydney, New South Wales, Australia). LA was considered positive when at least one of these tests confirmed its presence.

Immunoglobulin (Ig)G and IgM aCL were measured according to a standard aCL enzyme-linked immunosorbent assay (ELISA), as described elsewhere.[35]

IgG and IgM aβ2GPI were determined by ELISA method as previously reported.[36] Purified human β2GPI was purchased from Yamasa Corp. (Tokyo, Japan). Irradiated microtiter plates, Maxisorp (Nunc, Denmark) were coated with 4 μg/ml of purified β2GPI in phosphate-buffered saline (PBS) at 4°C and washed twice with PBS. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of 3% gelatin (BDH Chemicals Ltd, Poole, UK). After three washes with PBS containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) (PBS-Tween), 50 μl of serum diluted with PBS containing 1% bovine serum albumin (Sigma-Aldrich) (PBS-1% BSA) in 1:50 were added in duplicate. Plates were incubated for one hour at room temperature and washed three times with PBS-Tween. Fifty microliters per well of the appropriate dilution of alkaline phosphatase-conjugated goat anti-human IgG and IgM (Sigma-Aldrich) in PBS-1% BSA was added. After one hour of incubation at room temperature and after four washes in PBS-Tween, 100 μl/well of 1 mg/ml p-nitrophenylphosphate disodium (Sigma-Aldrich) in 1 M diethanolamine buffer (pH 9.8) were added. Following color development, optical density at 405 nm was measured by a Multiskan ascent plate reader (Thermo Electron Corporation, Waltham, MA, USA). Normal ranges of IgG (> 2.2 U/ml) and IgM (> 6.0 U/ml) aβ2GPI with cut-off values of 99th percentile were previously established using nonpregnant 132 healthy controls.

IgG and IgM aPS/PT were detected by ELISA, as previously described.[37] Briefly, nonirradiated microtiter plates (Sumilon Type S; Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of a 50 μg/ml preparation of phosphatidylserine (Sigma-Aldrich) and dried overnight at 4°C. To avoid nonspecific binding of proteins, the wells were blocked with 150 μl of Tris-buffered saline (TBS) containing 1% fatty acid-free BSA (catalog no. A6003; Sigma-Aldrich) and 5 mM $CaCl_2$ (BSA-$CaCl_2$). After three washes in TBS containing 0.05% Tween 20 (Sigma-Aldrich) and 5 mM $CaCl_2$ (TBS-Tween-$CaCl_2$), 50 μl of a 10 μg/ml preparation of human prothrombin (Diagnostica Stago) in BSA-$CaCl_2$ was added to half of the wells in the plates, and the same volume of BSA-$CaCl_2$ alone (as sample blank) was added to the other half. After one hour of incubation at 37°C, the plates were washed, and 50 μl of serum diluted 1:100 in BSA-$CaCl_2$ was added to duplicate wells. Plates were incubated for one hour at room temperature, and alkaline phosphatase-conjugated goat anti-human IgG or IgM and substrate were added. The aPS/PT antibody titer of each sample was derived from the standard curve according to dilutions of the positive control.

### *Genotyping*

CD36 gene polymorphisms were investigated in this population using the TaqMan polymerase chain reaction (PCR) genotyping method on a 7500 Fast Real-Time PCR System® (Applied Biosystems, Foster City, CA, USA). Genomic DNA samples were extracted from peripheral blood. Related risk for having APS or SLE was approximated by odds ratio (OR).

### *Materials*

Animal studies were reviewed and approved by Hokkaido University Institutional Animal Care and Use Committee. CD36KO mice were kindly donated by Dr Yamashita, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Japan.[38] FA6-152, a mouse monoclonal anti-human CD36 antibody (aCD36) with the CD36 signal blocking property, was purchased (Abcam, Cambridge, UK).[39] A mouse monoclonal aPS/PT with LA activity, 231D, was prepared as described previously.[3] Purified total IgG from APS patients either aCL- or aPS/PT-positive (Pt-aCL and Pt-aPS/PT, respectively) or healthy donors (Healthy-IgG) were prepared using MelonTM Gel IgG Purification Kit (Takara Bio, Ohtsu, Japan). Purity of IgG was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pt-aCL and Pt-aPS/PT were confirmed to have aPL titers by ELISA as described above. Clinical profiles of the patients whose IgG was purified are shown in Table 2. All antibodies were confirmed not to be contaminated with lipopolysaccharide (LPS) using Limulus ES-II Single Test® (Wako, Osaka, Japan).

### *Mouse peritoneal macrophage (MPM) stimulation assay*

At three days after intraperitoneal injection of 2 ml 10% proteose peptone (Becton Drive, Franklin

**Table 2** Clinical profiles of patients whose IgG was purified and used in our experiments

| *No* | *Disease* | *Age-Sex* | *Arterial thrombosis* | *Venous thrombosis* | *Pregnant morbidity* | *LA* | *aCL* | *aβ2GPI* | *aPS/PT* |
|---|---|---|---|---|---|---|---|---|---|
| Pt-aCL1 | PAPS | 52-F | Stroke | – | – | + | + | + | – |
| Pt-aCL2 | PAPS | 27-F | – | – | Miscarriage | + | + | + | – |
| Pt-aCL3 | PAPS | 46-F | Stroke | DVT | – | + | + | + | – |
| Pt-aCL4 | APS-SLE | 49-F | Stroke | DVT | Miscarriage | + | + | + | – |
| Pt-aCL5 | PAPS | 52-F | Stroke | – | – | + | + | + | – |
| Pt-aPS/PT1 | PAPS | 29-M | Stroke | DVT | – | + | – | – | + |
| Pt-aPS/PT2 | APS-SLE | 18-F | – | DVT | – | + | – | – | + |
| Pt-aPS/PT3 | APS-SLE | 25-F | Stroke | – | Miscarriage | + | – | – | + |
| Pt-aPS/PT4 | APS-SLE | 33-F | – | DVT | Miscarriage | + | – | – | + |
| Pt-aPS/PT5 | APS-SLE | 22-F | Stroke | PE | Miscarriage | + | – | – | + |
| Pt-aPS/PT6 | APS-SLE | 30-M | – | DVT | – | + | – | – | + |
| Pt-aPS/PT7 | APS-SLE | 45-F | Stroke | DVT | | + | – | – | + |

IgG: immunoglobulin G; F: female; M: male; LA: lupus anticoagulant; aCL: anticardiolipin antibodies; aβ2GPI: anti-β2 glycoprotein I antibodies; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PAPS: primary antiphospholipid syndrome; DVT: deep vein thrombosis; PE: pulmonary embolism.

Lakes, NJ, USA), MPM were harvested from 8- to 12-week-old female CD36KO or C57BL/6J wild-type (WT) mice. MPMs were suspended in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin and their concentration adjusted to $1 \times 10^6$ cells/ml. MPMs were then treated with aPL (300 μg/ml Pt-aCL, 300 μg/ml Pt-aPS/PT or 10 μg/ml 231D) and incubated for four hours at 37°C 5% $CO_2$. Pt-aPS/PT and 231D were used in the presence of 2.5 mM $CaCl_2$ and 10 μg/ml human prothrombin (Diagnostica Stago).[3] Equal concentrations of Healthy-IgG or mouse IgG1κ isotype control (Becton Drive) was used as a negative control and 600 ng/ml LPS (Sigma-Aldrich) was used as a positive control. Data were obtained by five or more independent experiments.

### *Human peripheral blood mononuclear cell (PBMC) stimulation assay*

Venous blood was collected in heparin from a healthy donor. Human PBMC were isolated on Ficoll-Paque plus® gradient centrifugation (Amersham Biosciences, Piscataway, NJ, USA). Human PBMCs were suspended in DMEM supplemented with 10% fetal calf serum containing penicillin and streptomycin and their concentration adjusted to $1 \times 10^6$ cells/ml. Human PBMC were then treated with aPL (200 μg/ml Pt-aCL, 200 μg/ml Pt-aPS/PT or 2 μg/ml 231D) in the presence or absence of 1 μg/ml aCD36 and incubated for four hours at 37°C 5% $CO_2$. Pt-aPS/PT and 231D were used in the presence of 2.5 mM $CaCl_2$ and 10 μg/ml human prothrombin. Equal concentrations of Healthy-IgG or mouse IgG1κ isotype control was used as a negative control and 1 ng/ml LPS was used as a positive control. Data were obtained by three or more independent experiments. The healthy donor was confirmed to have CD36 on both monocytes and platelets by analysis by flow cytometry before the experiments were performed (data not shown).

### *RNA extraction and quantitative TaqMan real-time PCR*

Total RNA was isolated from MPM or human PBMC using RNeasy Mini Kit® (Qiagen, Valencia, CA, USA) and reverse-transcribed with Super ScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative analysis of TF or IL-6 gene expression was performed by real-time PCR using 7500 Fast Real-Time PCR System® and gene-specific TaqMan Minor Groove Binder probes (Mm00438855m1, Hs01076032m1, Mm004461 90m1 and Hs00174131m1; Applied Biosystems). The level of the TF or IL-6 transcript was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase. Relative quantification was performed using the comparable cycle threshold method.

### *Monocyte TF antigen expression by flow cytometry*

Surface TF expression on human monocytes, treated with aPL as described above, was evaluated by flow cytometry with a direct double-color immunofluorescence technique. Resuspended human PBMC were incubated with phycoerythrin-conjugated mouse monoclonal anti-human CD14 (Beckman Coulter, Brea, CA, USA) and with fluorescein-conjugated mouse monoclonal anti-human

**Table 3** Allele frequencies of CD36 gene polymorphisms

| *Group* | *Minor allele frequency* | *P value* | *OR (95% CI)* |
|---|---|---|---|
| rs3765187 (C478T Pro90Ser) | (TT + TC vs CC) | | |
| Healthy subjects ($n = 422$) | 10.2% (43/422) | – | – |
| APS ($n = 132$) | 3.8% (5/132) | 0.032 | 0.35 (0.13 to 0.90) |
| SLE/non-APS ($n = 265$) | 7.9% (21/265) | 0.32 | 0.76 (0.44 to 1.31) |
| rs1049654 (on 5'UTR) | (C vs A) | | |
| Healthy subjects ($n = 416$) | 26.7% (222/832) | – | – |
| APS ($n = 123$) | 26.8% (66/246) | 0.96 | 1.01 (0.73 to 1.39) |
| SLE/non–APS ($n = 261$) | 28.7% (150/522) | 0.41 | 1.11 (0.87 to 1.41) |

*P* value and OR (95% CI) for each group were obtained by comparison with healthy subjects. OR (95% CI): odds ratio (95% confidence interval); UTR: untranslated region; APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus.

TF (Lifespan Biosciences, Seattle, WA, USA) for 30 minutes at 4°C. Cells were resuspended and fixed in 2% paraformaldehyde (Sigma-Aldrich). Analysis by flow cytometry was performed on an acoustic focusing cytometer (Attune; Applied Biosystems). Gating was accomplished using size, complexity and phycoerythrin gates to define the monocyte population of PBMC.

### *Statistical analysis*

Statistical evaluation was performed by chi square test, Fisher's exact test or Student's *t* test, as appropriate. *P* values less than 0.05 were considered significant.

## Results

### *Allele frequencies of CD36 gene polymorphisms*

Allele frequencies of the two CD36 gene polymorphisms were compared among three groups: healthy subjects, APS and SLE in the absence of APS. Minor allele carrier of rs3765187 (C478T Pro90Ser), a missense mutation linked to human CD36 deficiency, was significantly less frequent in APS (3.8%) compared to healthy subjects (10.2%). In contrast, rs3765187 minor allele carrier was as frequent in SLE in the absence of APS as it was in healthy subjects. There was no significant difference in allele frequency of rs1049654, a mutation on the 5' untranslated region, among those groups (Table 3).

### *Expressions of TF and IL-6 on MPM induced by aPL*

Expressions of TF and IL-6 were analyzed on MPM from WT or CD36KO mice cultured with each aPL and its antigen. All three aPL used in this experiment, Pt-aCL, Pt-aPS/PT and 231D, induced TF mRNA expression in MPM up to 10-fold. The aPL-induced TF mRNA expression was significantly reduced in MPM from CD36KO mice compared to MPM from WT mice (Figure 1(a)). Those three aPL induced IL-6 mRNA expression in MPM up to 60-fold. The aPL-induced IL-6 mRNA expression was significantly reduced in MPM from CD36KO mice compared to MPM from WT mice (Figure 1(b)).

TF: tissue factor; IL-6: interleukin 6; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; IgG: immunoglobulin G; MPM: mouse peritoneal macrophage; WT: wild-type; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; LPS: lipopolysaccharide.

### *Expressions of TF and IL-6 on human PBMC induced by aPL*

Expressions of TF and IL-6 were analyzed on human PBMC from a healthy donor cultured with each aPL and its antigen. All three aPL used in this experiment, Pt-aCL, Pt-aPS/PT and 231D, induced TF mRNA expression in human PBMC from a healthy donor up to 16-fold. The aCD36 significantly reduced aPL-induced TF mRNA expression in human PBMC. In contrast, equal concentration of mouse IgG1κ isotype control did not reduce it (Figure 2(a)). Those three aPL induced IL-6 mRNA expression in human PBMC from a healthy donor up to 40-fold. The aCD36 significantly reduced aPL-induced IL-6 mRNA expression in human PBMC. In contrast, equal concentration of the mouse IgG1κ isotype control did not reduce it (Figure 2(b)). We next performed analysis by flow cytometry to confirm the TF expression on monocytes. Those three aPL also induced surface TF expression on human

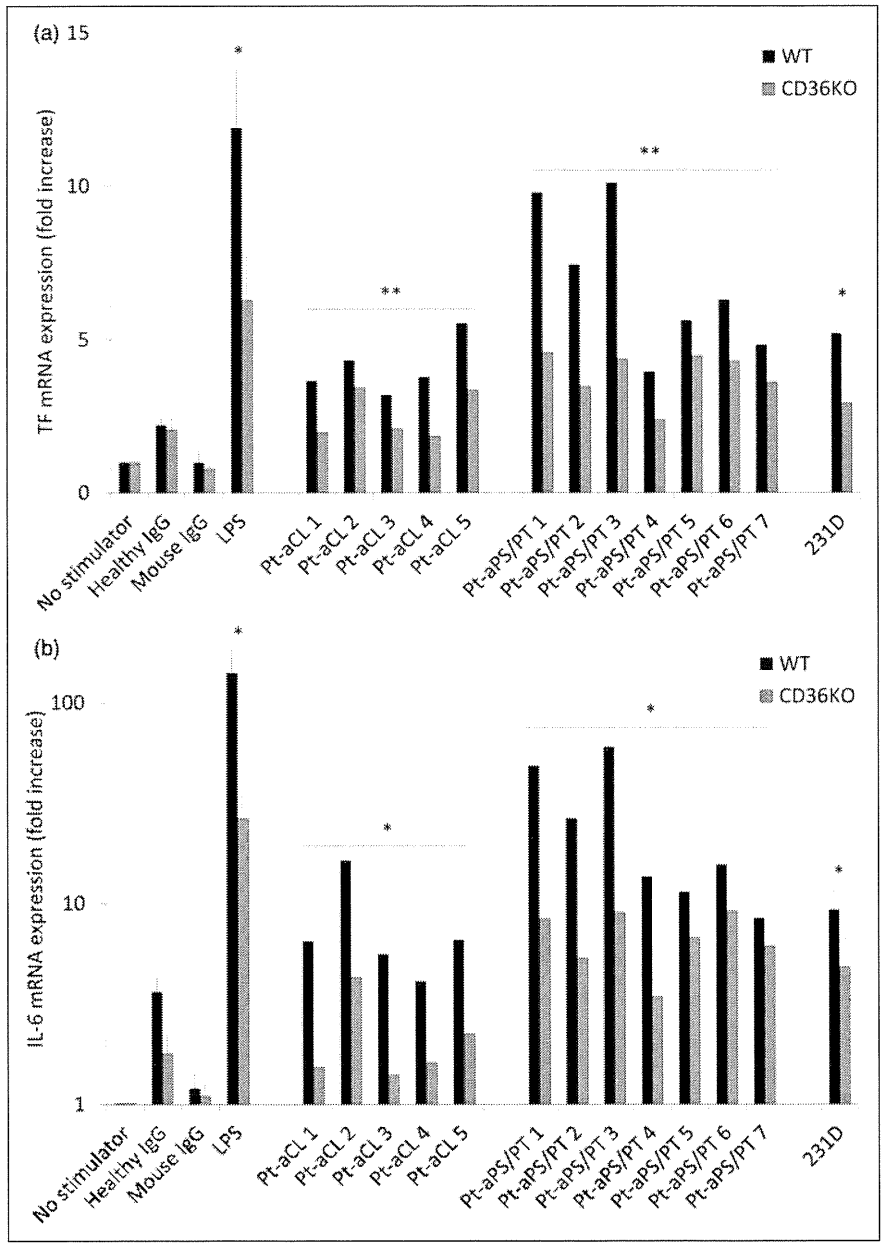


**Figure 1** Evaluation of TF (a) and IL-6 (b) mRNA levels induced by aPL in mouse peritoneal macrophages. Expressions of TF and IL-6 mRNA were analyzed in MPM from WT or CD36KO mice cultured with each aPL and its antigen. Healthy-IgG represent the mean of five healthy donors. The mRNA levels induced by Pt-aCL or Pt-aPS/PT are individually indicated for each patient. Values were normalized to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and expressed as fold increase in the Y axis. Error bars show standard errors of the mean obtained by five or more experiments. *: $p < 0.05$ and **: $p < 0.01$. *P* values were obtained by comparison between WT and CD36KO using Student's *t* test.
TF: tissue factor; IL-6: interleukin 6; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; IgG: immunoglobulin G; MPM: mouse peritoneal macrophage; WT: wild-type; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; LPS: lipopolysaccharide.

CD14-positive cells from a healthy donor. The aCD36 reduced aPL-induced surface TF expression on human CD14-positive cells. In contrast, equal concentration of the mouse IgG1κ isotype control did not reduce it (Figure 3).

aCD36: anti-CD36 antibody; TF: tissue factor; IL-6: interleukin 6; IgG: immunoglobulin G; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PBMC: human peripheral blood mononuclear cells; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/

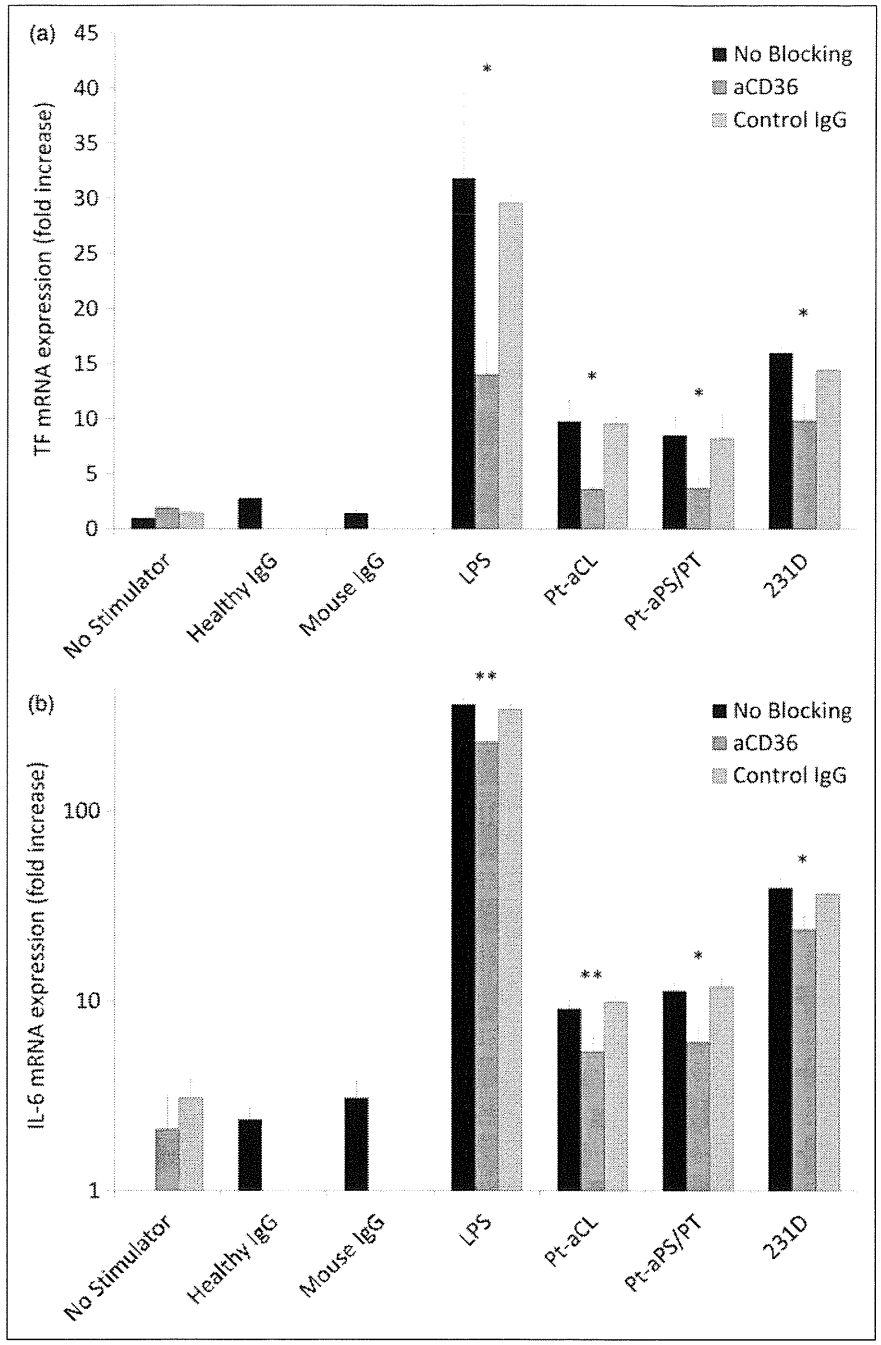


**Figure 2** Evaluation of TF (a) and IL-6 (b) mRNA levels induced by aPL in human PBMC. Expressions of TF and IL-6 mRNA were analyzed in human PBMC from a healthy donor cultured with each aPL and its antigen. Healthy-IgG represent the mean of three healthy donors. Pt-aCL and Pt-aPS/PT were both from a patient with primary APS (Pt-aCL5 and Pt-aPS/PT1 shown in Table 2), which induced the highest TF expression in the response of mouse peritoneal macrophages. Values were normalized to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and expressed as fold increase in the Y axis. Error bars show standard errors of the mean obtained by three or more experiments. *: $p < 0.05$ and **: $p < 0.01$. $P$ values were obtained by comparison between No blocking and aCD36 using Student's $t$ test.
aCD36: anti-CD36 antibody; TF: tissue factor; IL-6: interleukin 6; IgG: immunoglobulin G; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PBMC: human peripheral blood mononuclear cells; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

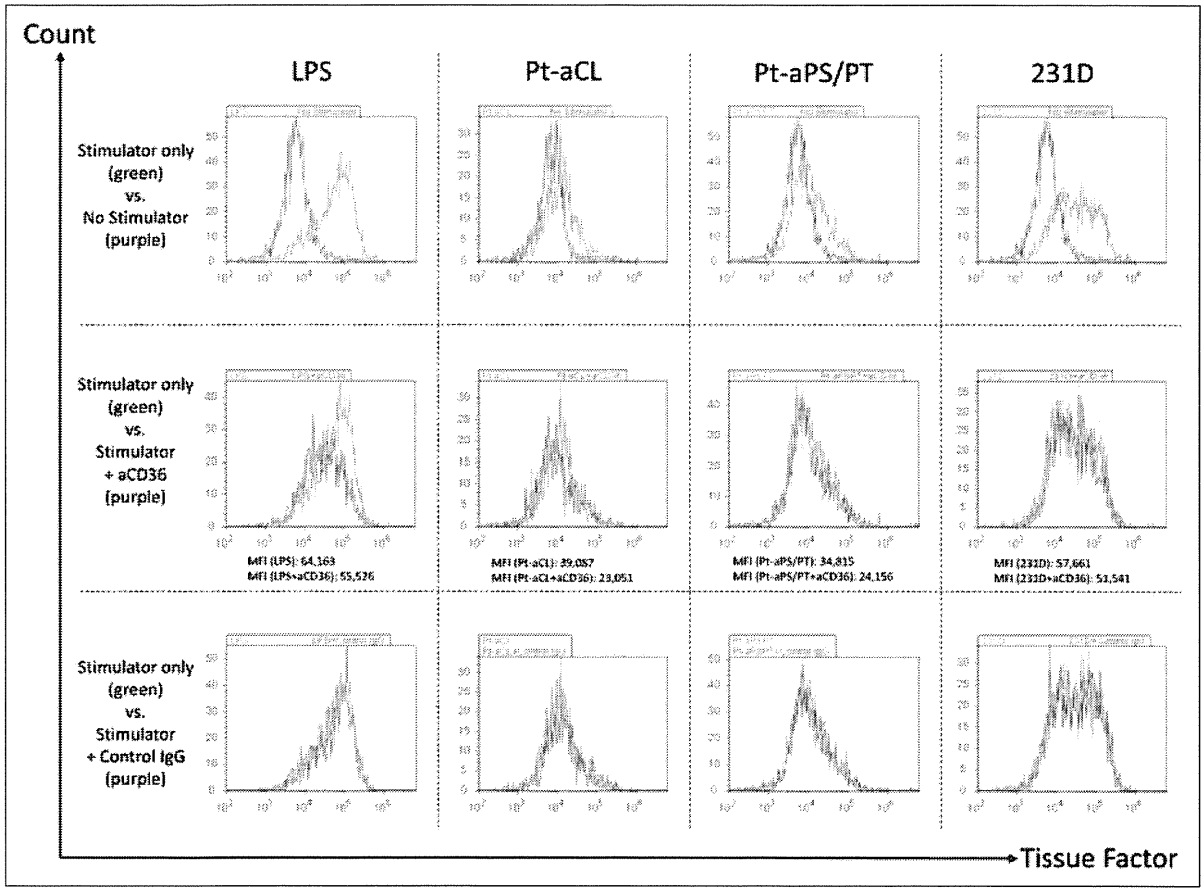


**Figure 3** Evaluation of TF protein level induced by aPL on human monocytes. Expression of TF protein was analyzed on human monocytes from a healthy donor cultured with each aPL and its antigen. Histogram plots show the TF expression on CD14-positive cells. Upper, middle and lower column show the comparison of stimulator only (green line) with no stimulator, blocking with aCD36 and blocking with control IgG (purple line), respectively. Pt-aCL and Pt-aPS/PT were both from a patient with primary APS (Pt-aCL5 and Pt-aPS/PT1 shown in Table 2), which induced the highest TF expression in the response of mouse peritoneal macrophages.
aCD36: anti-CD36 antibody; MFI: mean fluorescence intensity; TF: tissue factor; IgG: immunoglobulin G; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

PT: purified total IgG from aPS/PT-positive APS patient; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

aCD36: anti-CD36 antibody; MFI: mean fluorescence intensity; TF: tissue factor; IgG: immunoglobulin G; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

## Discussion

In this study, we demonstrated that the gene mutation linked to human CD36 deficiency was less frequent in patients with APS and that the deficient or suppressed CD36 function significantly reduced aPL-induced TF/IL-6 expressions in vitro. CD36 may be involved in the thrombotic pathophysiology in patients with APS. A few patients with APS, however, had the gene mutation linked to human CD36 deficiency and knocking out CD36 did not lead to complete diminuendo of aPL-induced TF expression. Taken together, CD36 may be one of the cell surface receptors involved in the pathogenesis of APS.

CD36 resides in lipid raft domains and interacts with a variety of membrane receptors, such as integrin $\alpha 3\beta 1$, $\alpha 6\beta 1$, tetraspanins and TLRs. The latest was elegantly demonstrated on macrophage in studies showing cooperation between CD36 and TLR2 or TLR6 in the recognition and response to

**Table 4** Clinical profiles of patients with APS who have minor allele of rs3765187 (C478T Pro90Ser)

| *No* | *n1478T* | *Disease* | *Age-Sex* | *Arterial thrombosis* | *Venous thrombosis* | *Pregnant morbidity* | *LA* | *aCL* | *aβ2GPI* | *aPS/PT* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Homo | PAPS | 32-F | – | CRVO | – | – | + | – | – |
| 2 | Hetero | PAPS | 42-F | – | – | Miscarriage | + | – | – | + |
| 3 | Hetero | APS-SLE | 23-F | Splenic infarct | DVT | – | + | + | + | + |
| 4 | Hetero | APS-SLE | 24-F | – | DVT | – | + | – | – | – |
| 5 | Hetero | APS-SLE | 49-F | Stroke | DVT | Miscarriage, eclampsia | + | + | – | + |

APS: antiphospholipid syndrome; LA: lupus anticoagulant; F: female; aCL: anticardiolipin antibodies; aβ2GPI: anti-β2 glycoprotein I antibodies; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PAPS: primary antiphospholipid syndrome; CRVO: central retinal vein occlusion; DVT: deep vein thrombosis.

bacteria cell wall components, such as *Staphylococcus*-derived lipoteichoic acid and diacylated lipoproteins.[31,40] Several CD36 functions, including microglial phagocytosis and platelet response, require integrin α3β1, α6β1 or tetraspanins.[41,42]

The mechanism of the thrombotic tendency in APS has been clarified at the molecular level by many investigations. TF upregulation on procoagulant cells is considered to be the most important procedure in the pathogenesis of APS. Elevation of plasma TF level and upregulation of TF expression on monocytes, which was accompanied by an increase in TF pathway inhibitor, were found in patients with APS.[6,43] Elevated plasma level of soluble fibrin and that of D-dimer, which reflects thrombin generation and fibrin turnover, were also found, presumably related to the "chronic" TF upregulation and activation of extrinsic coagulation pathway.[3] In in vitro studies, monocytes and endothelial cells treated with aPL demonstrated upregulation of TF and adhesion molecules.[5,6] NF-κB and p38MAPK were shown to participate in the procoagulant cell activation as intracellular signaling pathways. We[7] and others[8] showed that p38 MAPK protein was phosphorylated with NF-κB activation by aCL/β2GPI treatment and that SB203580, a specific p38 MAPK inhibitor, decreased the aCL/β2GPI-induced TF mRNA expression.

A number of candidates for the cell surface receptor involved in this pathogenesis have been reported.[9–16] Sorice et al.[44] showed the lipid raft recruitment of β2GPI and TLR-4 in human monocytes when interacting with aCL/β2GPI, suggesting that the procoagulant cell activation by aPL may involve the recruitment of cell surface receptors on lipid rafts. Given that CD36 resides in lipid raft domains and interacts with a variety of membrane receptors, our data support those findings and suggest that CD36 interacts with other β2GPI/prothrombin receptors involved in the pathogenesis of APS.

In clinical practice, treatment of APS has focused on utilizing antithrombotic medications such as warfarin, heparin or aspirin. Despite long-term antithrombotic medications, thrombosis can recur in patients with APS and antithrombotic medications can be associated with bleeding.[45] Given that thrombotic events occur only occasionally in patients with APS, aPL increase the thrombophilic threshold as the "first hit," and then clotting takes place only when a "second hit" exists, such as an infection or a surgical procedure.[46] Current antithrombotic medications in APS are directed to modulate the final event or "second hit." However, treatments that modulate the "first hit" would be more beneficial and potentially less harmful than current antithrombotic medications.

Our results suggest that inhibition or reduction of CD36 can be one of the options for the prophylaxis against thrombosis in patients with APS. Treatment targeting CD36 might be safe because heredity CD36 deficiency is not associated with serious clinical manifestations including bleeding disorders, suggesting that CD36 is a strong potential target of the treatment of patients with APS. CD36 expression is regulated by multiple agents on monocytes. It can be upregulated by adhesion, macrophage-colony stimulating factor, granulocyte/macrophage-colony stimulating factor, native and modified LDL, cellular cholesterol, IL-4 and high glucose conditions, while downregulated by corticosteroids, transforming growth factor-β1, high-density lipoprotein and LPS.[47] Statin and cilostazol, medical agents having some pleiotropic effects, were reported to downregulate CD36 expression on monocytes.[48,49] These agents might have implications for treatment of APS.

Given that CD36 deficiency may be protective for developing APS, we suspected some specific clinical features in patients with APS who have

the minor allele rs3765187. In our study, one patient with APS who carried the homozygous minor allele of rs3765187 exhibited central retinal vein occlusion as a sole APS manifestation and had aCL as a sole aPL; on the other hand, four heterozygous carriers exhibited typical APS manifestations and serological abnormalities (Table 4). Further studies will better delineate the correlation between minor allele of rs3765187 and severity of APS manifestations.

In conclusion, both genetically and biologically, our results suggest that in a susceptible background CD36 scavenger receptor function may be involved in the thrombotic pathophysiology in patients with APS.

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## Conflict of interest

The authors have no conflicts of interest to declare.

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# Prediction of Response to Treatment by Gene Expression Profiling of Peripheral Blood in Patients with Microscopic Polyangiitis

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## Abstract

The JMAAV study was an open-labeled prospective clinical trial, which proposed severity-based treatment protocols for patients with microscopic polyangiitis (MPA). The results suggest that the proposed protocols are useful (remission rate: 89.4%), but are also indicative of relapse or patient demise regardless of the treatment (recurrence rate: 19.0%; mortality rate: 10.6%). The aim of this study is to develop the method to predict response to the treatment in patients with MPA. In the present study, transcriptome analysis was performed using peripheral blood from patients enrolled in the JMAAV study before and 1-week after the beginning of treatment. The gene expression profile before treatment was not directly related to the response to the treatment. However, when the samples from 9 patients with good response (persistent remission for 18 months) were examined, the expression of 88 genes was significantly altered by the treatment. Thirty statistically reliable genes were selected, and then the alteration of expression by the treatment was examined among 22 patients, including 17 with good response, which was defined as persistent remission for 18 months and 5 with poor response, which was defined as relapse after remission or no remission. Discrimination analysis between the alteration of expression of the 30 genes by the treatment and the response identified a combination of 16 genes as the most valuable gene set to predict the response to the treatment. This preliminary study identified IRF7, IFIT1, IFIT5, OASL, CLC, GBP-1, PSMB9, HERC5, CCR1, CD36, MS4A4A, BIRC4BP, PLSCR1, DEFA1/DEFA3, DEFA4, and COL9A2 as the important genes that can predict the response to the treatment in patients with MPA at an early point during the therapy.

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## Introduction

The spectrum of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) includes microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, Churg-Strauss syndrome), and granulomatosis with polyangiitis (GPA, Wegener's granulomatosis) [1]. The two major antigens of ANCA are myeloperoxidase (MPO) [2] and proteinase 3 (PR3) [3]. MPO-ANCA is often detected in the sera of patients with MPA and EGPA; while, PR3-ANCA is a useful marker for GPA. Although it remains unsolved why ANCA is produced, immunological mechanisms are considered to be involved in the development of AAV. Therefore, corticosteroids and immunosuppressive agents have been used as treatments for AAV. Based on previous clinical trials, the standard protocol of treatment for AAV was established in Western countries [4–6].

The prevalence of MPA is strikingly higher in Japanese population compared to the Caucasoid [7]. Accordingly, clinical trials to establish a guideline for the management of patients with this subtype of AAV should be held in Japan. Ozaki and colleagues instituted a Japanese study group for MPA and conducted an open-labeled prospective clinical trial, the JMAAV study (The University Hospital Medical Information Network, Clinical Trials Registry; http://www.umin.ac.jp/ctr/index-j.htm, registration number ID 000000867) [8]. In the JMAAV study, patients newly diagnosed with MPA were stratified into 3 categories based on