Title	The involvement of CD36 in monocyte activation by antiphospholipid antibodies
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Citation	Lupus, 22(8), 761-771 https://doi.org/10.1177/0961203313490242
Issue Date	2013-07
Doc URL	http://hdl.handle.net/2115/54688
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Type	article (author version)
File Information	Lupus_22(8)_761-771.pdf

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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 β 2 glycoprotein I-dependent anticardiolipin antibodies (aCL/ β 2GPI) and phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) ^{2, 3}.

Both antibodies recognize epitopes on the phospholipid-binding proteins (β 2GPI 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 1990s, the function of phospholipid-binding protein was extensively studied based on the hypothesis that the interaction of aPL to their antigens impairs their anti- or pro-coagulant activities. However, the activation of procoagulant cells (monocyte, endothelial cell and platelet) through the binding of phospholipid-binding protein and aPL has been focused to be investigated 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 or other proinflammatory substances ^{5, 6}. Enhanced TF expression has been observed in healthy monocytes incubated with polyclonal or monoclonal aPLs ⁶. The activation of nuclear factor- κ B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK) has been recognized to participate in this process as intracellular signaling pathways ^{7, 8}. However, the cell surface receptor to participate in this process is still controversial, and a number of candidates have been reported including annexin A2, apolipoprotein E receptor 2', glycoprotein Ib α , 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 α 5 β 1 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- κ B ^{18, 19}. Human CD36 deficiency was first described in 1989 in subjects refractory to HLA-matched platelet transfusions ²⁰ and is found in 4 to 10 % of Asian or African populations ²¹⁻²³. It is divided into 2 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

Japanese population (rs3765187, C478T, Pro90Ser)²¹, a nonsense mutation linked to human CD36 deficiency in African population (rs3211938, T1264G)²⁵, and a mutation on 5' untranslated region (rs1049654) whose clinical significance is unknown. Some clinical phenotypes of CD36 deficiency have been reported including hypertrophic cardiomyopathy, hypertension and dyslipidemia²⁶⁻²⁸. The phenotypes of CD36 knock out (KO) mice have been reported to be protective to 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, 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, activated partial thromboplastin time and dilute Russell's viper venom time, were performed for lupus anticoagulant determination 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. IgG and IgM aCL were measured according to a standard aCL ELISA, as described elsewhere ³⁵. IgG and IgM a β 2GPI ³⁶ and aPS/PT ³⁷ were determined by ELISA method as previously reported. Expanded methods are available in the online data supplement.

Genotyping

CD36 gene polymorphisms were investigated in this population using TaqMan polymerase chain reaction genotyping method on 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.

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 Japan³⁸. FA6-152, a mouse monoclonal anti-human CD36 antibody (aCD36) with the CD36 signal blocking property, was purchased (Abcam, Cambridge, UK)³⁹. 231D, a mouse monoclonal aPS/PT with lupus anticoagulant activity, was prepared as described previously³. 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 Melon™ 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 enzyme-linked immunosorbent assay 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 stimulation assay

At 3 days after intraperitoneal injection of 2 ml 10% proteose peptone (Becton Drive, Franklin Lakes, NJ, USA), mouse peritoneal macrophages (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 adjusted their concentration to 1×10^6 cells/ml. MPMs were then treated with aPL (300 μ g/ml Pt-aCL, 300 μ g/ml Pt-aPS/PT or 10 μ g/ml 231D) and incubated 4 hours at 37°C 5% CO₂. Pt-aPS/PT and 231D were used in the presence of 2.5 mM CaCl₂ and 10 μ g/ml human prothrombin (Diagnostica Stago) ³. Equal concentrations of Healthy-IgG or mouse IgG1 κ isotype control (Becton Drive) was used as a negative control and 600 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) was used as a positive control. Data were obtained by five or more independent experiments.

Human peripheral blood mononuclear cell stimulation assay

Venous blood was collected in heparin from a healthy donor. Human peripheral blood mononuclear cells (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 adjusted their concentration to 1×10^6 cells/ml. Human PBMCs were then treated with aPL (200 μ g/ml Pt-aCL, 200 μ g/ml Pt-aPS/PT or 2 μ g/ml 231D) in the presence or absence of 1 μ g/ml aCD36 and incubated 4 hours at 37°C 5% CO₂. Pt-aPS/PT and 231D were used

in the presence of 2.5 mM CaCl₂ 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 experiments (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 Script™ 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, Mm00446190m1 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 done 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-colour immunofluorescence technique. Resuspended human PBMCs were incubated with phycoerythrin-conjugated mouse monoclonal anti-human CD14 (Beckman Coulter, Brea, CA, USA) and with fluorescein-conjugated mouse monoclonal anti-human TF (Lifespan Biosciences, Seattle, WA, USA)

for 30 min 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 5' untranslated region, among those groups (Table 3).

Expressions of TF and IL-6 on mouse peritoneal macrophages induced by antiphospholipid antibodies

Expressions of TF and IL-6 were analyzed on MPM from WT or CD36KO mice cultured with each aPL and its antigen. All three aPLs 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 not completely but significantly reduced in MPM from CD36KO mice compared to MPM from WT mice (Figure 1A). Those three aPLs induced IL-6 mRNA expression in MPM up to 60-fold. The aPL-induced IL-6 mRNA expression was not completely but significantly reduced in MPM from CD36KO mice compared to MPM from WT mice (Figure 1B).

Expressions of TF and IL-6 on human peripheral blood mononuclear cells induced by antiphospholipid antibodies

Expressions of TF and IL-6 were analyzed on human PBMC from a healthy donor cultured with each aPL and its antigen. All three aPLs 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 IgG1 κ isotype control did not reduce it (Figure 2A). Those three aPLs 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 mouse IgG1 κ isotype control did not reduce it (Figure 2B). We next performed analysis by flow cytometry to confirm the TF expression on monocytes. Those three aPLs also induced surface TF expression on human 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 mouse IgG1 κ isotype control did not reduce it (Figure 3).

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 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 $\alpha 3\beta 1$, $\alpha 6\beta 1$ or tetraspanins^{41, 42}.

The mechanism of the thrombotic tendency in APS has been clarified at the molecule level by many researches. 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

tissue factor 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 aPLs demonstrated upregulation of TF and adhesion molecules ^{5,6}. NF- κ B 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- κ B activation by aCL/ β 2GPI treatment and that SB203580, a specific p38 MAPK inhibitor, decreased the aCL/ β 2GPI-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 β 2GPI and TLR-4 in human monocytes when interacting with aCL/ β 2GPI, 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 β 2GPI/prothrombin receptors involved in the pathogenesis of APS.

In clinical practice, treatment of APS has been focused on utilizing anti-thrombotic medications such as warfarin, heparin or aspirin. Despite long-term anti-thrombotic mediations, thrombosis can recur in patients with APS and anti-thrombotic mediations 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 procedure⁴⁶. Current anti-thrombotic medications in APS are directed to modulate the final event or ‘second hit’. However, treatments that modulate ‘first hit’ would be more beneficial and potentially less harmful than current anti-thrombotic 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 hereditary CD36 deficiency does not associate with serious clinical manifestations including bleeding disorders, suggesting that CD36 is a strongly 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 β 1, high density lipoprotein and LPS⁴⁷. 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 minor allele of rs3765187. In our study, one patient with APS who carried 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 (Supplementary table). 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.

Acknowledgements

The authors thank Dr. Yamashita, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine Japan for CD36KO mice donation and Miki Aoto for their technical support in the performance of some laboratory tests. This work was supported by the Japanese Ministry of Health, Labour and Welfare, and the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

Funding

Olga Amengual is funded by MEXT and the Japanese Society for the Promotion of the Science (project number 21-40106).

Conflict of interest

The authors have no conflicts of interest to declare.

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Table 1. Profiles of patients with antiphospholipid syndrome

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)	
LAC	107
aCL	73
a β 2GPI	66
aPS/PT	92

LAC: lupus anticoagulant, aCL: anticardiolipin antibodies, a β 2GPI: anti- β 2 glycoprotein I antibodies, aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies

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	LAC	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		+	-	-	+

LAC: 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.

Table 3. Allele frequencies of CD36 gene polymorphisms

rs3765187 (C478T Pro90Ser)	Minor allele carrier frequency (TT+TC vs CC)	P value	OR (95% CI)
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)	Minor allele frequency (C vs A)	P value	OR (95% CI)
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.

Count

LPS

Pt-aCL

Pt-aps/PT

231D

Stimulator only
(green)
vs.
Stimulator
+ aCD36
(purple)

MFI (LPS): 64,163
MFI (LPS+aCD36): 55,526

Pt-aCL Pt-aCL+aCD36

MFI (Pt-aCL): 39,087
MFI (Pt-aCL+aCD36): 23,051

Pt-apsPT Pt-apsPT+aCD36

MFI (Pt-aps/PT): 34,815
MFI (Pt-aps/PT+aCD36): 24,156

231D 231D+aCD36

MFI (231D): 57,661
MFI (231D+aCD36): 51,541

Stimulator only
(green)
vs.
Stimulator
+ Control IgG
(purple)

Pt-aCL Pt-aCL+Control IgG

Pt-apsPT Pt-apsPT+Control IgG

231D 231D+Control IgG

Tissue Factor

Figure 1. Evaluation of TF (A) and IL-6 (B) mRNA levels induced by antiphospholipid antibodies 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.

Figure 2. Evaluation of TF (A) and IL-6 (B) mRNA levels induced by antiphospholipid antibodies in human peripheral blood mononuclear cells.

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.

Figure 3. Evaluation of TF protein level induced by antiphospholipid antibodies 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 shows 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.