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Tissue factor expression on monocytes induced by anti-phospholipid antibodies as a strong risk factor for thromboembolic complications in SLE patients

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Abstract

Our aim was to clarify the role of anti-phospholipid antibodies in the pathogenesis of monocyte tissue factor (TF) expression and thromboembolic complications (TE) in patients with SLE. We examined cell surface expression of TF on monocytes in 93 SLE patients. Monocyte TF expression was significantly higher in SLE patients who had TE than in other SLE patients, and confirmed that the high expression of monocyte TF was a strong risk factor for TE. Furthermore, the presence of anti-cardiolipin/β2-glycoprotein I antibodies (anti-CL/β2-GPI) was strongly associated with the high expression of monocyte TF. We therefore studied the in vitro effect of IgG anti-CL/β2-GPI on lipopolysaccharide (LPS)-induced expression of TF on monocytes in healthy peripheral blood and found that purified IgG containing anti-CL/β2-GPI significantly enhanced LPS-induced monocyte TF expression. These results suggest that anti-CL/β2-GPI cause persistently high TF expression on monocyte, which may contribute to the risk of thromboembolic events in SLE patients.

Keywords: Systemic lupus erythematosus; Anti-phospholipid syndrome; Monocyte; Tissue factor; Anti-cardiolipin antibodies; Thromboembolic complications; Enzyme-linked immunosorbent assay; Flow cytometry

Anti-phospholipid antibodies (aPLs) are a heterogeneous group of autoantibodies that appear in a variety of autoimmune diseases, particularly systemic lupus erythematosus (SLE) [1,2]. Anti-cardiolipin/β2-glycoprotein I antibodies (anti-CL/β2-GPI), which is detected by an enzyme-linked immunosorbent assay (ELISA), and lupus anticoagulant activity (LA), which is detected by a phospholipid-dependent coagulation assay, are the most common and best characterized aPLs [3–5]. These antibodies are associated with clinical events such as arterial and/or venous thromboembolic complications (TE) and obstetric complications [6–8]. Anti-phospholipid syndrome (APS) is defined by both clinical findings (recurrent arterial and/or venous thrombosis and recurrent fetal loss) and laboratory evidence of persistent aPLs (anti-CL/β2-GPI and/or LA activity) [9]. However, the precise mechanism responsible for TE in APS patients remains unclear.

It is well known that aPLs do not bind to the negatively charged phospholipid itself but instead bind to complexes of the phospholipid and plasma proteins such as β2-GPI

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and prothrombin [2]. It was recently reported that human monocytes synthesize β2-GPI and that β2-GPI expression on monocytes is increased in SLE patients with aPLs [10], suggesting that β2-GPI expressed on the monocyte surface is a physiopathologically relevant target for anti-CL/β2-GPI. Furthermore, it has been shown that β2-GPI expression on the monocyte surface in APS patients is closely related to tissue factor (TF) expression on the monocyte surface [10]. TF is a cell surface-anchored glycoprotein that binds both zymogen factor VII (FVII) and the active serine protease factor VIIa (FVIIa). The TF/FVIIa complex activates coagulation by cleaving its substrates, factors IX and X, ultimately leading to thrombin generation, fibrin deposition, and platelet activation. In a normal state, TF is not expressed on cells that are in contact with flowing blood because TF is an integral membrane protein constitutively expressed in many cell types outside the vasculature [11]. However, TF is expressed on endothelial cells and monocytes in response to endotoxin, TNF, and some cytokines. Monocytes have a potent procoagulant activity when TF is expressed on their surface. Expression of TF on monocytes has been suggested as a mechanism of thrombosis in several conditions, including sepsis, atherosclerosis, cancer, and the use of oral contraceptives [12,13].

Recently, the presence of anti-CL/β2-GPI was reported to be associated with increased levels of TF messenger RNA (mRNA) synthesis and monocyte TF activity, and up-regulation of monocyte TF expression induced by anti-CL/β2-GPI has been hypothesized to be a possible mechanism of aPLs-associated thrombotic complications [14,15]. In the present study, we examined TF expression on the monocyte surface by flow cytometric analysis using anti-human TF monoclonal antibodies and anti-CD14 monoclonal antibodies in 93 SLE patients with or without arterial and/or venous TE, and we investigated whether high TF expression on monocyte caused by anti-CL/β2-GPI is related to the pathogenesis of TE in patients with SLE.

Materials and methods

Patients. We studied 93 patients (90 females, 3 males; age range, 8–76 years; mean 39.8 years) with SLE diagnosed according to the revised criteria of The American Rheumatism Association. Clinical histories revealed TE in 35 SLE patients including 18 cases of arterial TE and 17 cases of venous TE. All of the TE had been documented by venography, arteriography, angiography, Doppler ultrasound, and/or computed tomography scan. Patients who had any congenital abnormality with respect to antithrombin, protein C, protein S, or Factor V Leiden were excluded from the study. Informed consent was obtained from all patients.

As controls, we also tested 47 whole blood samples from normal healthy volunteers (37 females, 10 males; age range, 22–74 years; mean, 44.4 years). None of the volunteers had a history of thrombosis, and no abnormalities in blood cell count, coagulation, liver function, or autoimmune activity were found.

Whole blood assay for monocyte TF expression. Monocyte TF expression was assessed by measurement of surface expression of human TF in CD14-positive cells by flow cytometric analysis. Peripheral blood samples were collected taken in vacuum tubes containing EDTA-2K (2.0 mL total volume; Sekisui), and an aliquot (100 μL) of EDTA-anticoagulated whole blood was added to a falcon tube containing 5 μL of phycoerythrin (PE)-conjugated anti-human CD14 (Dako Cytomation) and 10 μL of fluorescein isothiocyanate (FITC)-conjugated anti-human TF (#4508CJ; American Diagnostica). As a negative control, we added 10 μL of FITC-conjugated mouse IgG_1 (Dako Cytomation) in place of anti-human TF. All tubes were immediately incubated for 20 min at room temperature in the dark. We performed the steps of hemolysis and fixation by using an automatic system (TQ-PrepTM, Beckman Coulter). Each sample was hemolyzed by adding 600 μL of formic acid (1.2 mL/L) and vortexing for 8 s, and then added sodium carbonate (6 g/L), sodium chloride (14.5 g/L), and sodium sulfate (31.3 g/L) and vortexed each tube for 10 s to stop the hemolysis. The sample in each tube was fixed by adding 100 μL of paraformaldehyde (10 g/L). This fixation method resulted in complete lysis of the red blood cells. After we added 2 mL of phosphate-buffered saline (PBS) to each sample, we washed the sample by centrifuging for 5 min at 600*g*, aspirating the supernatant, and resuspending the pellet in 4 mL of PBS. This wash step was performed two times. The pellet was resuspended in 500 μL of PBS and then examined by flow cytometry.

Flow cytometric analysis. The flow cytometer was calibrated daily for particle size, particle fluorescent intensity, and laser beam alignment with Calibrates beads (Flow-Check, Fluorospheres, Beckman Coulter). During calibration these parameters were adjusted so that the coefficient of variation (CV) would be <2%. Flow cytometric acquisition and analysis of 5000 monocytes was performed with a Beckman Coulter FC500 cytometer. Monocytes were defined as CD14-positive cells. TF expression was measured as the percentage of CD14-positive cells with staining of TF. This was achieved by plotting the side scatter characteristics vs CD14. The negative and positive delineator was determined by gating 1% background staining on the FITC-conjugated mouse IgG1 control. The percentage of FITC (TF)-positive events in this population was then determined.

Detection of anti-CL/β2-GPI. The presence of aPLs was determined on the basis of measurements of anti-CL/β2-GPI. Blood samples were collected in vacuum tubes (5.0-mL total volume, Sekisui, Japan) containing 0.5 mL of 3.13 g/L trisodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$), and platelet-poor plasma was prepared by double centrifugation at 2800*g* for 15 min. The concentrations of anti-CL/β2-GPI were measured with a standard ELISA (Yamasa Shoyu Co., Ltd.) as reported previously [16]. β2-GPI dependency of anti-CL/β2-GPI activity was tested as previously described [16].

Plasma samples from 150 normal healthy volunteers (staff members of Osaka University Hospital; 62 female, 88 male; age range, 21–58 years; mean, 40.8 years) were used as control samples for levels of anti-CL/β2-GPI. The concentrations of anti-CL/β2-GPI in 150 normal control plasma samples, detected by ELISA, were log transformed with the Stat Flex program to approximate normality before statistical analysis were performed. The concentration of 3.0 U of anti-CL/β2-GPI, which was the mean + 6SD of the normal controls, was considered the cut-off point for normal in the present study. Results were considered positive when the anti-CL/β2-GPI level exceeded the cut-off value.

IgG purification. The IgG fractions were purified from plasma samples (anti-CL/β2-GPI-positive patients, $n = 5$; anti-CL/β2-GPI-negative patients, $n = 4$; normal pooled plasma, $n = 4$) with Protein G Sepharose (Pharmacia LKB Biotechnology). The purity of the IgG was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The final concentration of IgG used in our study was 4 g/L. A pool of commercially available human IgG (Immuno AG) was used as a control.

Effect of IgG fractions from plasma with or without anti-CL/β2-GPI on monocyte TF expression. After preparation of each IgG fraction, we studied its effect on TF expression on the surface of normal monocytes in vitro. Normal peripheral blood samples (90 μL of EDTA-whole blood) were incubated for 120 min at room temperature with 10 μL of purified IgG fraction (0, 1, 2, or 4 g/L) from the anti-CL/β2-GPI-positive patients, anti-CL/β2-GPI-negative patients, or normal pooled plasma. Then l0 μL of lipopolysaccharide (LPS, 10 mg/L, final concentration) was added to induce minimum TF expression. Because it was recently reported that expression of TF mRNA in response to LPS stimulation peaked at 120 min, we incubated our samples at 37 °C for precisely 120 min. At

exactly 120 min after the start of LPS stimulation, 5 μL of PE-conjugated anti-human CD14 and 10 μL of FITC-conjugated anti-human TF was added to each sample tube. As a negative control, we added 10 μL of FITC-conjugated mouse IgG1 in place of anti-human TF. All tubes were immediately incubated 20 min at room temperature in the dark. Each sample was then fixed as described above. After fixation of cells, the sample was washed twice with 4 mL PBS and examined by flow cytometry.

Statistical analysis. We used the Kruskal–Wallis test and the non-parametric Mann–Whitney test to compare the percentage of TF-positive monocytes between SLE patients with TE, SLE patients without TE, and healthy blood donors. As an approximation of the relative risk, we calculated the odds ratios (ORs) and 95% confidence intervals (CIs) for several putative risk factors by multivariate logistic regression analysis with the statistical program Stat Flex (Ver. 4.2; Arthch Inc.). We considered an OR statistically significant when the lower limit of the 95% CI was >1.0.

Results and discussion

Although an association between the presence of aPLs and the prevalence of TE in patients with SLE has been established, the precise mechanism underlying thrombosis in these patients remains unclear. In the present study, we confirmed that anti-CL/β2-GPI causes persistently high expression of TF on monocytes, and this may be involved in the pathogenesis of TE in SLE patients.

Many studies have suggested that up-regulation of monocyte TF expression induced by aPLs is a significant contributor towards the hypercoagulability associated with APS [15]. Increased monocyte procoagulant activity in SLE patients with aPLs was first reported by de Prost et al. [17]. Moreover, it was reported that patients with primary APS have increased expression of TF on the monocyte surface, along with increased levels of TF mRNA in peripheral blood mono-nuclear cells [18,19]. Subsequently, a number of studies suggested that plasma, purified IgG, and anti-β2-GPI antibodies from APS patients enhance TF expression and procoagulant activity on normal monocytes [20–23]. Additionally, several of these studies demonstrated that anti-β2-GPI human monoclonal antibodies derived from peripheral B cells of APS patients enhance monocyte TF activity and levels of TF mRNA in a β2-GPI-dependent fashion [20,23]. Roubey et al. demonstrated that anti-β2-GPI antibodies are at least one specificity involved in inducing monocyte TF, by using an anti-β2-GPI monoclonal antibody and affinity-purified anti-β2-GPI autoantibodies [24]. Furthermore, recent experimental evidence suggested that aPLs induces TF gene and protein expression in monocytes from APS patients by simultaneously and independently activating the phosphorylation of p38 MAP kinases and nuclear translocation and activation of NF-κB/Rel proteins, and the phosphorylation of MEK-1/ERK proteins [25,26].

However, the association between up-regulation of monocyte TF expression induced by anti-CL/β2-GPI and the prevalence of arterial and/or venous TE in patients with SLE were not established clearly. To investigate whether high TF expression on monocyte caused by anti-CL/β2-GPI is related to the pathogenesis of TE in patients with SLE, we examined TF expression on the monocyte

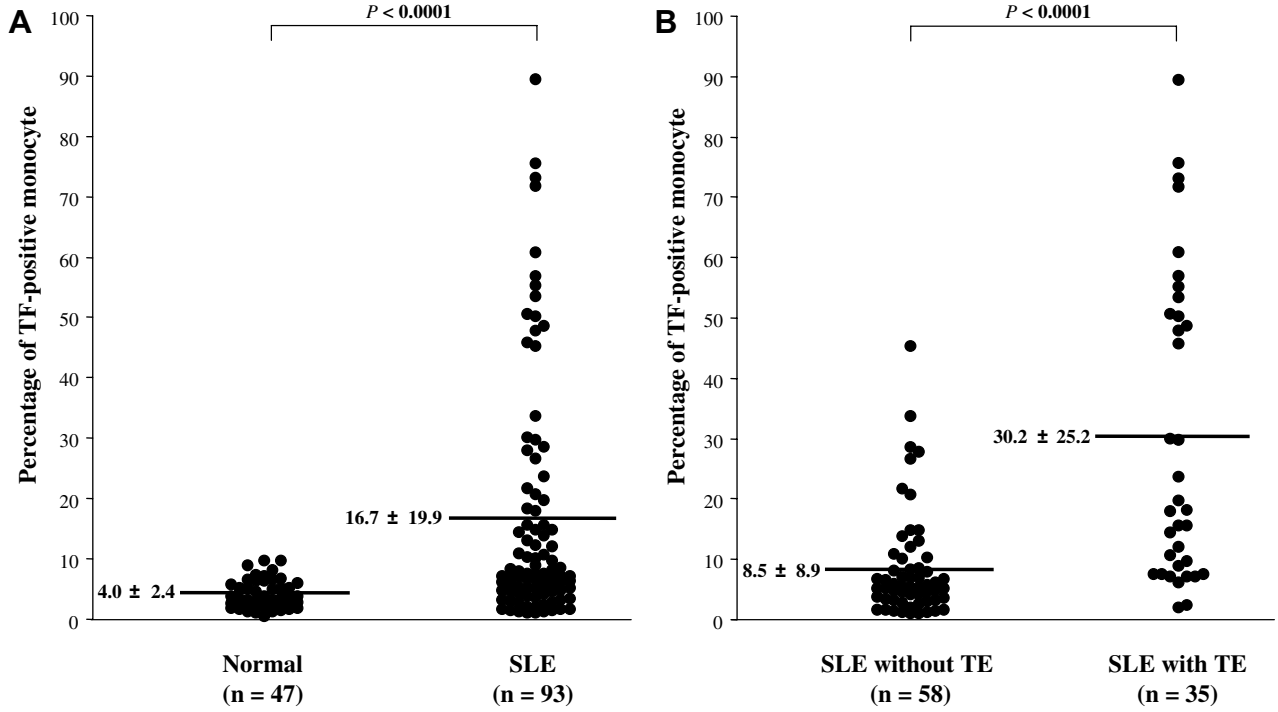

Fig. 1. TF expression on the surface of monocytes (percentage of TF-positive monocytes), as detected by flow cytometric analysis with anti-human TF and anti-CD14 monoclonal antibodies. (A) Comparison of TF expression on monocytes between 47 normal healthy control subjects and 93 SLE patients. (B) Comparison of TF expression on monocytes between 58 SLE patients without thromboembolic complications (TE) and 35 SLE patients with TE. Results are expressed as means ± SD.

surface by flow cytometric analysis in 93 SLE patients and 47 healthy subjects. Monocyte TF expression, which was defined as the percentage of TF-positive monocytes, was significantly higher in SLE patients than that in healthy blood donors (Fig. 1A). Furthermore, when SLE patients were divided into two groups (35 with TE and 58 without TE), the percentage of TF-positive monocytes in SLE patients with TE was significantly higher than in those without TE (Fig. 1B).

The mean + 3SD (11.2%) of the percentage of TF-positive monocytes in the 47 controls was chosen as the cut-off point for normal in the present study. We regarded a result as high TF expression when the percentage of TF-positive monocytes exceeded this cut-off point. The prevalence of TE was significantly higher in patients with high expression of monocyte TF than those in patients with normal expression of monocyte TF. Multivariate logistic analysis of the risk for TE revealed that the high expression of monocyte TF was a strong risk factor for TE (Table 1).

When we evaluated the prevalence of anti-CL/β2-GPI in 93 SLE patients, anti-CL/β2-GPI was detected in 26 (28.0%) of these patients, and we confirmed that the presence of anti-CL/β2-GPI was strongly associated with the high expression of monocyte TF (Table 2). However, plasma of SLE patients might contain components other than anti-CL/β2-GPI that could affect monocyte TF expression. Moreover, circulating monocytes in SLE patients might overexpress TF in response to stimulation of other factor(s). Therefore, we examined the in vitro effects of IgG fraction purified from plasma of SLE patients with or without anti-CL/β2-GPI on TF expression by normal monocytes. Incubation of normal resting peripheral blood cells with purified IgG fraction from plasma of anti-CL/β2-GPI-positive patients did not significantly induce expression of TF on monocytes in our preliminary study. Because the antigenic targets recognized by

Table 1
Association between monocyte TF expression and thromboembolic complications

	TF expression		Multivariate logistic analysis		
	High (>11.2%)	Normal (0–11.2%)	OR	95% CI	*P* value
TE-positive	23 (65.7%)	12 (20.7%)	7.35	2.86–18.9	<0.0001
TE-negative	12 (34.3%)	46 (79.3%)			

TF, tissue factor; TE, thromboembolic complications; OR, odds ratio. CI, confidence intervals.

Table 2
Association between monocyte TF expression and anti-CL/β2-GPI

TF expression	Anti-CL/β2-GPI		Multivariate logistic analysis		
	Positive (>3.0 U)	Negative (0–3.0 U)	OR	95% CI	*P* value
High (>11.2%)	20 (76.9%)	15 (22.4%)	13.0	4.40–38.4	<0.0001
Normal (0–11.2%)	6 (23.1%)	52 (77.6%)			

Anti-CL/β2-GPI, anti-cardiolipin/β2-glycoprotein I antibodies; TF, tissue factor; OR, odds ratio; CI, confidence intervals.

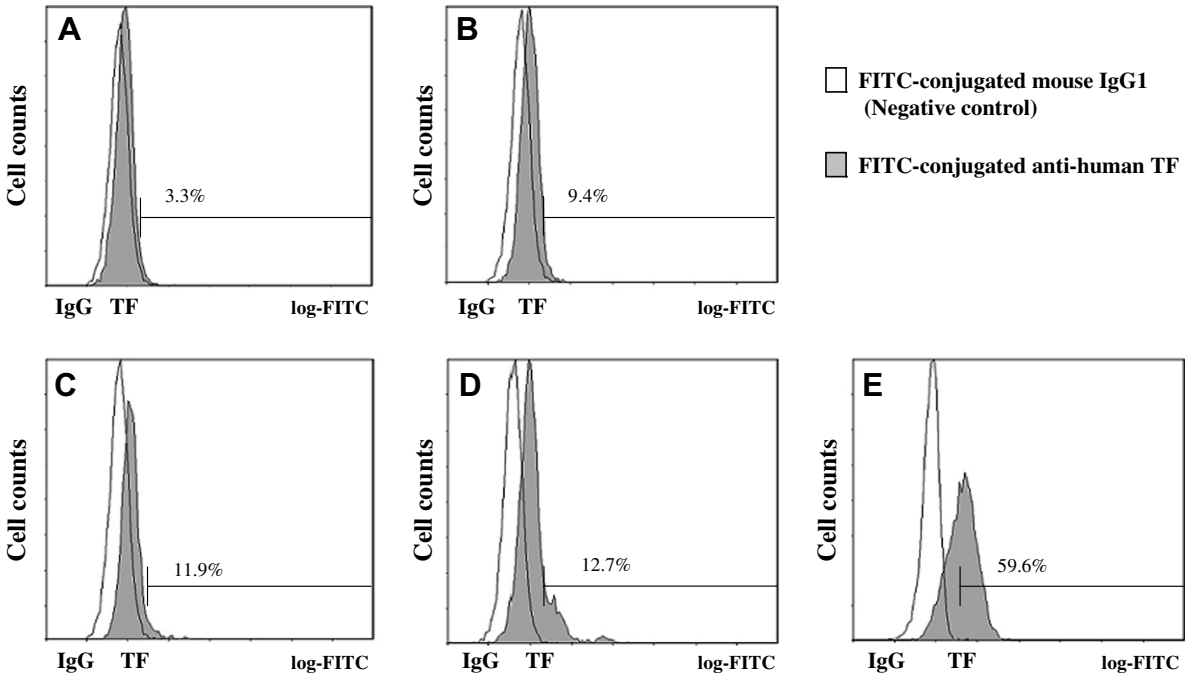

Fig. 2. Representative data of flow cytometric analysis of monocyte TF expression in response to LPS stimulation. (A) Unstimulated normal monocytes. (B) Monocytes stimulated with LPS (10 mg/L, final concentration) for 120 min. (C) Monocytes pre-incubated with the IgG fraction purified from normal pooled plasma. (D) Monocytes pre-incubated with the IgG fraction purified from plasma of anti-CL/β2-GPI-negative patients. (E) Monocytes pre-incubated with the IgG fraction purified from plasma of anti-CL/β2-GPI-positive patients.

anti-CL/β2-GPI are not expressed on the surface of normal resting monocytes, anti-CL/β2-GPI may not bind to such monocytes. Recent reports indicated that human monocytes can synthesize β2-GPI and that monocytes may increase β2-GPI exposure on their surface after stimulation with various substances [10]. These findings suggest that β2-GPI expressed on the monocyte surface is an antigenic target for anti-CL/β2-GPI. Furthermore, it was reported that β2-GPI expression on the monocyte surface in APS patients is closely related to monocyte TF expression in these patients [10]. Therefore, we examined whether IgG fractions purified from the plasma of anti-CL/β2-GPI-positive patients could enhance the expression of TF on monocytes in response to LPS. Representative data from flow cytometric analysis are shown in Fig. 2. The percentage of TF-positive monocytes in unstimulated normal peripheral blood cells was always less than 8.0%. (mean ± SD, 3.4 ± 1.6%, $n = 10$, Fig. 2A). When cells were stimulated with LPS (10 mg/L, final concentration) for 120 min at 37 °C, the percentage of TF-positive monocytes increased to 9.3 ± 2.4% (mean ± SD, $n = 10$) as show a representative case in Fig. 2B, but the values were always below 16.0%. Pre-incubation of normal resting peripheral blood cells with the purified IgG fractions from normal pooled plasma or from plasma of anti-CL/β2-GPI-negative patients did not significantly change the percentage of monocytes expressing TF in response to LPS stimulation (Fig. 2C and D, respectively). In contrast, pre-incubation of cells with purified IgG fractions from plasma of anti-CL/β2-GPI-positive patients increased the percentage of monocytes that expressed TF in response to LPS stimulation, as shown in a representative case (Fig. 2E). The percentages of TF-positive monocytes after stimulation with 10 mg/L of LPS for 120 min and pre-incubation with varying concentrations (0, 1, 2, or 4 g/L) of purified IgG for 120 min at room temperature are shown in Fig. 3. All purified IgG containing anti-CL/β2-GPI ($n = 5$) significantly enhanced LPS-induced monocyte expression of TF in a concentration-dependent manner. The percentage of TF-positive monocytes was 20.3 ± 5.5% (mean ± SD) after pre-incubation with 1.0 g/L IgG, 32.9 ± 8.0% with 2.0 g/L IgG, and 47.5 ± 13.3% with 4.0 g/L IgG. However, the purified IgG fractions from plasma of anti-CL/β2-GPI-negative patients ($n = 4$) or from normal pooled plasma ($n = 4$) did not significantly change LPS-induced expression of TF on monocytes surface. These findings suggest that high TF expression on monocyte induced by anti-CL/β2-GPI is directly contributed to the risk of arterial and/or venous TE in patients with SLE.

Because TF exposure on monocytes in SLE patients is thought to be induced frequently by infection, inflammation, or endothelial cell damage, circulating monocytes in SLE patients with anti-CL/β2-GPI may overexpress TF in response to stimulation of anti-CL/β2-GPI. The increased TF expression of circulating monocytes caused by anti-CL/β2-GPI may be important mechanism of arterial and/or venous TE in SLE patients.

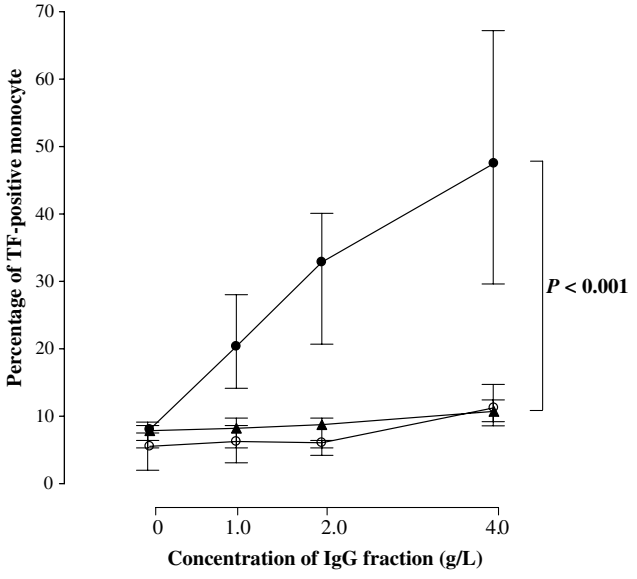

Fig. 3. Effects of IgG fractions purified from plasma of patients with or without anti-CL/β2-GPI on monocyte TF expression in response to LPS stimulation. The percentage of TF-positive monocytes in response to stimulation with 10 mg/L of LPS for 120 min after pre-incubation with varying concentrations (0, 1, 2, or 4 g/L) of purified IgG fraction for 120 min at room temperature are shown. (○) IgG fraction purified from normal pooled plasma ($n = 4$). (▲) IgG fraction purified from plasma of anti-CL/β2-GPI-negative patients ($n = 4$). (●) IgG fraction purified from plasma of anti-CL/β2-GPI-positive patients ($n = 5$). Results are expressed as means ± SD.

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