phy (MabTrap-TMGII; Amersham Pharmacia Biotech, Uppsala, Sweden).

Establishment of a mouse monoclonal anti-PS/PT antibody using prethrombin 1 as antigen. Prothrombin (1 mg/ml in TBS) was digested for 3 hours at 37°C with 10 units of bovine thrombin (Sigma). The reaction was stopped by the addition of 1 m*M* p-ABSF and p-APMSF. Prethrombin 1, which lacks the prothrombin domain 1 that comprises the phospholipid-binding site (Gla-domain), was purified from the solution by ion-exchange chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of prethrombin 1 fractions revealed a single band at 50 kd under nonreducing conditions.

To obtain a monoclonal APT that binds to prothrombin but does not interact with the phospholipid-binding site of prothrombin, a BALB/c mouse was immunized with prethrombin 1. Hybridomas were screened using an anti-PS/PT ELISA, and monoclonal antibody 51A6 was established and purified in the same manner as described for 231D.

APT-alone assay for activity of the monoclonal anti-PS/PT antibody. An APT-alone assay was performed as described previously (20), with some modifications. Briefly, either irradiated microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) or nonirradiated plates (Sumilon Type S) were coated overnight at 4°C with 10 μg/ml of purified human prothrombin in TBS containing 5 m*M* $CaCl_2$. Wells were blocked for 1 hour at 37°C with 0.5% gelatin. After 3 washes with TBS–Tween–$CaCl_2$, 50 μl of sample (monoclonal antibodies, control mouse IgG, or serum from mouse immunized with human prothrombin), diluted in BSA–$CaCl_2$ as appropriate, was added to duplicate wells. Plates were incubated for 1 hour at room temperature, followed by the addition of alkaline phosphatase–conjugated goat anti-mouse IgG and substrate. Optical density at 405 nm was then measured.

Detection of LAC activity in normal plasma using monoclonal anti-PS/PT antibody. Blood samples from 4 healthy donors were collected in precooled tubes containing a one-tenth volume of 0.105*M* sodium citrate and were immediately centrifuged at 2,000*g* for 15 minutes. Platelets were removed by filtration, and the platelet-free plasma was stored at −80°C. Different concentrations of monoclonal antibodies (50–3.1 μg/ml) were spiked into the pooled normal plasma, and clotting times were determined using the Start 4 system. Measurements of dRVVT and APTT were performed as described above. In addition, the kaolin clotting time (KCT) was measured with a kaolin solution (Dade-Behring) according to standard protocols.

Competitive ELISAs. IgG from 9 APS patients with high titers of anti-PS/PT antibodies was purified using protein G–Sepharose affinity chromatography (MabTrap-TMGII). Monoclonal antibody 231D or 51A6 (200 or 20 ng/ml) was added to plates that had been coated with PS/PT complex, and the plates were incubated for 1 hour at room temperature. Purified IgG (1 mg/ml) was added to the wells, and binding to PS/PT complex was determined by anti-PS/PT ELISA. The inhibition of IgG binding by monoclonal antibodies was calculated by comparing the optical density values with the values for IgG binding in the absence of monoclonal antibodies.

An additional competitive ELISA was performed in which 200 ng/ml of either 231D or 51A6 was coincubated with several concentrations (200, 50, 12.5, and 3.1 μg/ml) of 2 representative purified IgG from APS patients.

Measurement of in vitro thrombin generation. The effects of anti-PS/PT antibodies on thrombin generation were evaluated with a chromogenic assay, using the prothrombinase complex phospholipid, $CaCl_2$, purified human activated factor V (FVa; Haematologic Technologies, Essex Junction, VT), and FXa (Enzyme Research Laboratories). The thrombin generation assays used in this study were based on our previous analyses (21). Thrombin generation was measured by using a specific substrate for thrombin, D-Phe-pipecolyl-Arg-paranitroaniline (S-2238; Chromogenix Instrumentation Laboratory, Milan, Italy). Cephalin (PTT-Reagent RD; Roche Diagnostics, Basel, Switzerland), a phospholipid from rabbit brain extract, was used as the source of phospholipid. Cephalin was used at a dilution of 1:63 in assay buffer (1% BSA, 0.1 m*M* $CaCl_2$, TBS).

Ten microliters of a 10 μg/ml preparation of purified human prothrombin diluted in assay buffer, 10 μl of diluted phospholipid, and 40 μl of 231D at various concentrations was transferred into each well of a 96-well microtiter plate and then incubated at 37°C for 20 minutes. Ten microliters of FVa (0–1 ng/ml) and FXa (0.5–5 μg/ml) was added to the preincubated mixture, and the plate was left at room temperature for 2 minutes. The coagulation reaction was initiated by adding 25 μl of a 50 m*M* concentration of $CaCl_2$, followed by 25 μl of 2 m*M* concentration of S-2238. After incubation at 37°C, the absorbance of the mixture was measured at 405 nm with a Multiscan Ascent plate reader (Thermo Electron Corporation, Waltham, MA).

Statistical analysis. Statistical evaluation was performed by Mann-Whitney U test, Fisher's exact test, or Student's *t*-test, as appropriate. *P* values less than 0.05 were considered significant.

RESULTS

Plasma levels of thrombin generation and markers of fibrinolytic turnover. Levels of all markers of thrombin generation and fibrinolytic turnover were higher in APS patients with anti-PS/PT antibodies as compared with those in healthy control subjects. The distribution of representative markers, soluble fibrin antigen, and D-dimer are displayed in Figure 1. Plasma levels of soluble fibrin antigen and D-dimer were higher in both aCL subgroups of anti-PS/PT–positive patients as compared with those in healthy controls.

The cutoff level of each marker was defined as the mean ± 2SD of the levels in control subjects. A higher prevalence of elevation in the levels of markers of thrombin/plasmin generation (F_{1+2}, thrombin–antithrombin III complex, soluble fibrin antigen, D-dimer, and fibrin/fibrinogen degradation products) was found in all anti-PS/PT–positive patients, in anti-PS/PT–positive patients with aCL, and in anti-PS/PT–positive patients without aCL as compared with the levels in healthy subjects ($P < 0.05$ for each comparison) (Table 1).

Table 1. Prevalence of markers of increased thrombin/plasmin generation in patients and healthy controls*

	All patients	Anti-PS/PT+ patients		Healthy controls
		aCL+	aCL−	
Prothrombin fragment F_{1+2}	10/28 (36)	1/7 (14)	9/21 (43)	3/60 (5)
Thrombin–antithrombin III complex	10/36 (28)	2/14 (14)	8/22 (36)	7/73 (10)
Soluble fibrin antigen	16/36 (44)	8/14 (57)	8/22 (36)	6/127 (5)
D-dimer	18/36 (50)	8/14 (57)	10/22 (45)	1/73 (1)
Fibrin/fibrinogen degradation products	10/36 (28)	6/14 (43)	4/22 (18)	3/74 (4)

* Values are the number positive/total number tested (%). All values were statistically significant as compared with those in the controls ($P < 0.05$). Anti-PS/PT = anti–phosphatidylserine/prothrombin complex; aCL = anticardiolipin antibody.

Binding activity of mouse monoclonal anti-PS/PT antibody. Two anti-PS/PT antibody clones, 231D and 51A6, were obtained. The 231D antibody was established from a mouse that had been immunized with human prothrombin, and the 51A6 antibody was established from a mouse that had been immunized with

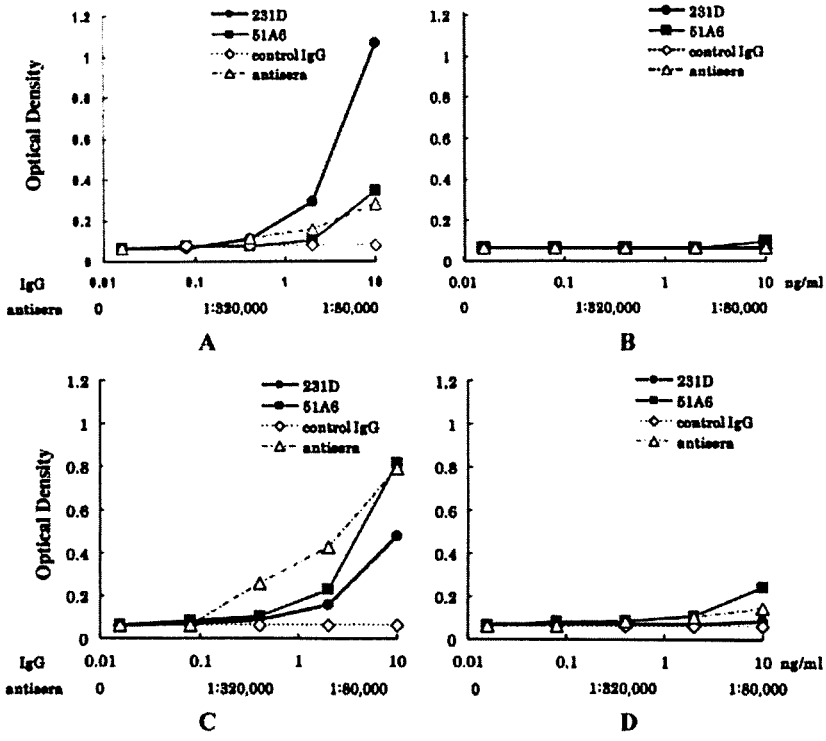

Figure 2. Binding activity of mouse monoclonal anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibodies 231D and 51A6. **A** and **B**, Binding activity of 231D and 51A6 for the PS/PT complex (**A**) and for phosphatidylserine alone (**B**) was determined by enzyme-linked immunosorbent assay (ELISA) using phosphatidylserine-coated plates. **C** and **D**, Binding activity of 231D and 51A6 for antiprothrombin antibody (APT), using prothrombin alone as the antigen coated onto either irradiated (**C**) or nonirradiated (**D**) plates, was determined by ELISA. Monoclonal antibody 51A6 bound to prothrombin coated on both irradiated and nonirradiated plates, whereas 231D showed little binding to prothrombin under both conditions. In all experiments, control IgG, consisting of purified mouse IgG from pooled normal mouse serum, was used at the indicated concentrations (ng/ml), and antisera, consisting of sera from mice that had been immunized with human prothrombin, were used at the indicated dilutions. Values are from a representative experiment.

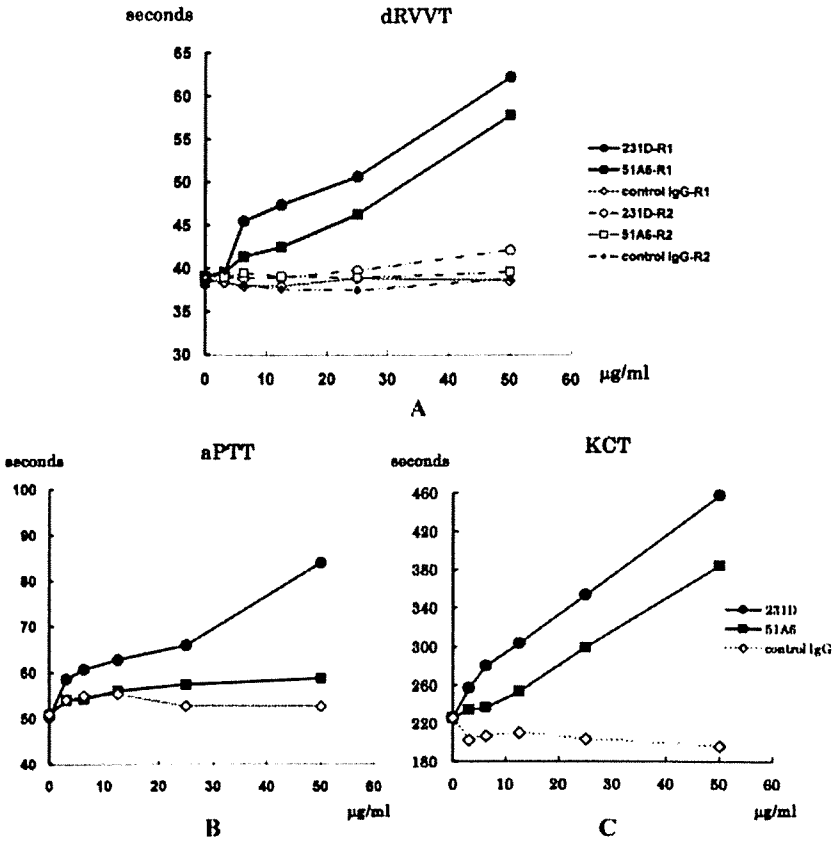

Figure 3. Lupus anticoagulant (LAC) activity of mouse monoclonal anti-phosphatidylserine/prothrombin complex antibodies 231D and 51A6, as determined by **A**, the dilute Russell's viper venom time (dRVVT), **B**, the activated partial thromboplastin time (APTT), and **C**, the kaolin clotting time (KCT). For measurement of dRVVT, purified 231D or 51A6 monoclonal antibody was added to normal plasma, and the dRVVT was determined. Reagent 1 (R1) contains a low concentration of phospholipid, and reagent 2 (R2) contains a high concentration of phospholipid. The APTT and KCT were determined in plasma that had been spiked with either 231D or 51A6. In all experiments, control IgG consisted of purified mouse IgG from pooled normal mouse serum. Numbers across the bottom are the concentration (in μg/ml) of 231D, 51A6, and control IgG tested. Values are from a representative experiment.

human prethrombin 1. Both clones bound strongly to the PS/PT complex, but not to phosphatidylserine alone (Figures 2A and B). Both murine monoclonal antibodies are of IgG1 isotype.

APT-alone activity was also investigated in the monoclonal anti-PS/PT antibodies. We found that 51A6 bound to prothrombin coated onto both irradiated and nonirradiated ELISA plates, but 231D showed a lower level of binding to prothrombin under both conditions (Figures 2C and D). Normal mouse IgG and pooled sera obtained from mice that had been immunized with human prothrombin were used as the negative control and the positive control, respectively.

LAC activity of monoclonal anti-PS/PT antibody. Purified 231D or 51A6 monoclonal antibody was added to normal plasma, and the dRVVT was measured in the monoclonal anti-PS/PT antibody–spiked plasma (Figure 3). With reagent 1 of the dRVVT test, which has a low phospholipid concentration, the clotting time of 231D-spiked plasma was prolonged in a dose-dependent manner. The clotting time was largely more prolonged with reagent 1 of the dRVVT test than with reagent 2, which

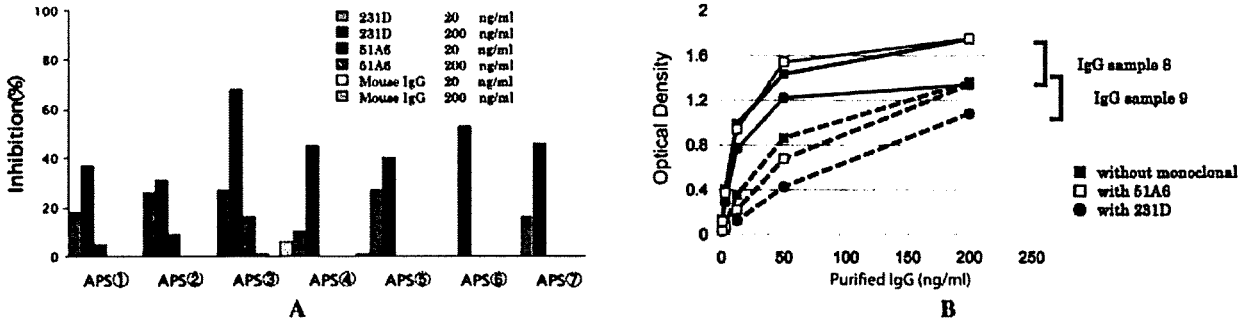

Figure 4. Competitive enzyme-linked immunosorbent assay (ELISA). IgG was purified from serum samples obtained from 9 patients with antiphospholipid syndrome (APS) who had anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibodies. **A,** Percentage inhibition of IgG binding in the presence of monoclonal antibody 231D, 51A6, or mouse IgG. Monoclonal antibody 231D, 51A6, or purified mouse IgG from pooled normal mouse serum (20 or 200 ng/ml) was preincubated on plates that had been coated with PS/PT complex, and 1 mg/ml of purified IgG from 7 of the APS patients was added. The percentage inhibition of IgG binding was calculated by comparing the optical density values in the presence of 231D, 51A6, or mouse IgG with the optical density values in the absence of 231D, 51A6, or mouse IgG, respectively. **B,** Inhibition curves following coincubation of monoclonal antibody 231D or 51A6 with the indicated concentrations of purified IgG from APS patients 8 and 9. As controls, purified IgG from the 2 APS patients was also incubated without monoclonal antibody.

contains a high concentration of phospholipids. Similar results were obtained with the 51A6-spiked plasma, but the clotting time was not as prolonged as with the 231D-spiked plasma.

Dose-dependent prolongations of the clotting time in both the APTT and the KCT tests were also found in 231D-spiked plasma and in 51A6-spiked plasma. Plasma containing 231D showed stronger anticoagulant properties than did plasma containing 51A6.

Findings of competitive ELISAs. The binding of purified IgG from anti-PS/PT–positive APS patients to the PS/PT complex was inhibited by 231D (35–70%). In contrast, there was no significant effect of 51A6 on the binding of IgG fractions to PS/PT complex (Figure 4A). Coincubation of 231D with purified IgG from APS patients also produced dose-dependent inhibition (Figure 4B).

Effects of monoclonal anti-PS/PT antibody on thrombin generation. The effect of monoclonal anti-PS/PT antibody on thrombin generation in vitro was evaluated by chromogenic assay using purified human clotting factors (Figures 5A–C). In the absence or in the presence of a very low concentration of FVa (0.1 ng/ml), the 231D monoclonal antibody increased thrombin generation by as much as 87% and in a dose-dependent manner. In contrast, when a high concentration of FVa (1 ng/ml) was added, 231D decreased thrombin generation by as much as 35%. The 51A6 monoclonal antibody displayed a lower level of inhibition of thrombin generation regardless of the concentration of FVa.

We also examined whether various concentrations of FXa altered the effects of 231D on thrombin generation (Figure 5D). Under 2 different constant concentrations of FVa, the effects of 231D on thrombin generation were increased in the presence of increasing concentrations of FXa. Again, we found that the 51A6 monoclonal antibody exhibited little inhibition of thrombin generation under any condition examined.

DISCUSSION

In this study, we demonstrated that the plasma levels of markers of thrombin generation/fibrinolysis turnover were elevated in patients with anti-PS/PT antibody, regardless of the coexistence of aCL. The mouse monoclonal anti-PS/PT antibody 231D, which has binding properties similar to those of anti-PS/PT found in patients with APS, showed "bipolar" effects on thrombin generation triggered by FXa.

Despite the proposal by some investigators of a possible correlation between APT and thrombosis, no clinical data have reported a link between increased thrombin/plasmin generation and antibodies against prothrombin. This study is the first to show the up-regulation of thrombin/plasmin generation in patients with anti-PS/PT antibody regardless of the presence of aCL. We also tested anti-β_2GPI antibodies in this study (data not shown), and the results were almost identical to those found in aCL. (None of our patients were positive for anti-β_2GPI antibodies but negative for aCL.)

There are several reports showing enhanced thrombin generation and fibrinolytic turnover in APS

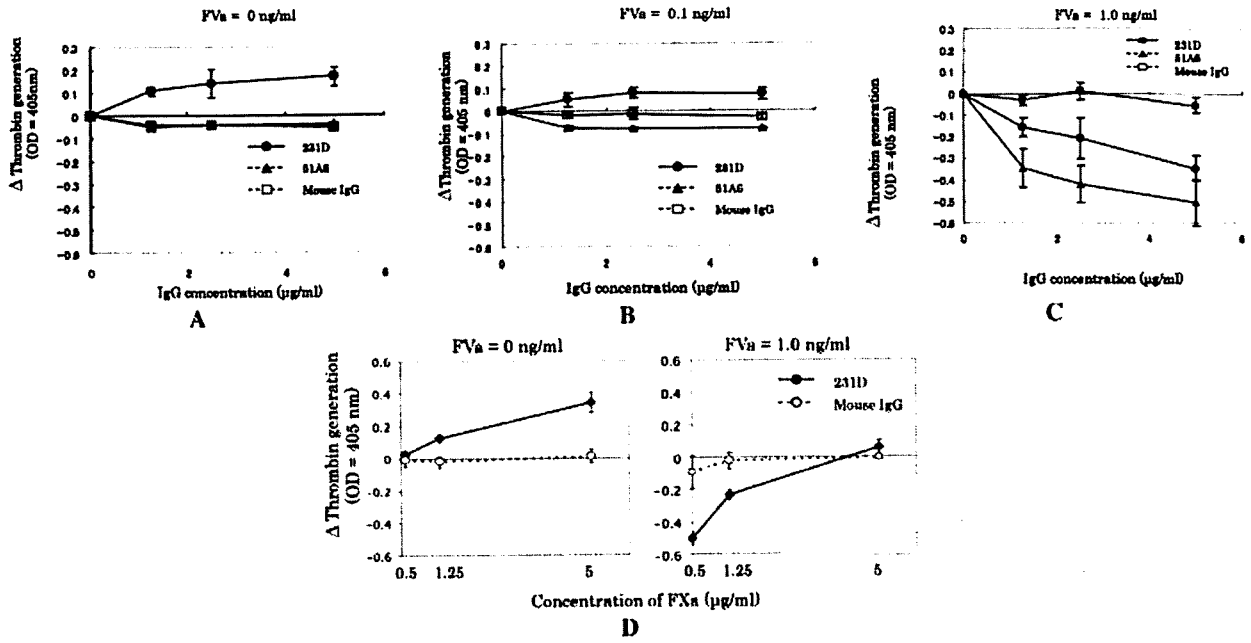

Figure 5. Evaluation of the effects of monoclonal anti–phosphatidylserine/prothrombin complex (anti-PS/PT) antibodies on thrombin generation in vitro, as determined by chromogenic assay. Purified human clotting factors were used in these experiments. **A–C**, Thrombin generation was measured in the absence of activated factor V (FVa) (**A**), in the presence of 0.1 ng/ml of FVa (**B**), and in the presence of 1 ng/ml of FVa (**C**), using a constant concentration of 1.25 μg/ml of FXa. **D**, Thrombin generation was measured in the absence of FVa (left) and in the presence of 1 ng/ml of FVa (right), using 0.5, 1.25, and 5 μg/ml of FXa. A constant concentration of 2.5 μg/ml of 231D or control IgG (purified mouse IgG from pooled normal mouse serum) was used. Values are the mean ± SEM difference in thrombin generation, as determined by the optical density at 405 nm (OD_{405}) value minus the OD_{405} value in the absence of monoclonal antibodies.

patients with aCL (22,23). In addition, de Laat et al (24) showed that β_2GPI-dependent LAC is highly correlated with thrombosis in patients with APS. Those reports clearly indicated that antibodies against β_2GPI, represented by aCL, anti-β_2GPI antibodies, or β_2GPI-dependent LAC, are correlated with high levels of thrombin generation. In contrast, there has been no report showing a correlation between antibodies against prothrombin, represented by prothrombin dependent LAC or anti-PS/PT. De Laat et al (24) failed to demonstrate increased thrombin generation in patients with β_2GPI-independent LAC, but such LAC would comprise antibodies against prothrombin and antibodies against nonspecific (or undetermined) proteins. Our data revealed that in the absence of antibodies against β_2GPI, levels of anti-PS/PT antibody correlated with elevated levels of markers of thrombin generation in APS patients, providing evidence that the increased thrombin/fibrin generation in these patients is related to anti-PS/PT itself.

The plasma samples were collected at least 3 months after the last thrombotic event, suggesting that patients with anti-PS/PT antibody are basically in a thrombophilic state. Some prothrombotic triggers may alter the balance between thrombin generation and regulators of thrombin generation, eventually leading to thrombosis.

The antibody responsible for prothrombin-dependent LAC activity is closely related to APTs detected by anti-PS/PT assay. In the setting of autoimmune disease, both anti-PS/PT and APT alone have been shown to be correlated with the presence of LAC, but anti-PS/PT had a markedly stronger relative risk for the presence of LAC than did APT alone (16). Many patients in that study had both anti-PS/PT and APT alone, but no correlation of their titers was found, even though some patients had very high levels of anti-PS/PT antibody in the absence of APT alone and vice versa.

To clarify the characteristics and properties of anti-PS/PT in thrombin generation or in the prothrombotic state observed in patients with anti-PS/PT, we successfully established 2 monoclonal antibodies. The

231D monoclonal antibody, which was obtained by immunizing mice with whole prothrombin, showed strong anti-PS/PT activity. Prothrombin was digested with thrombin, and the prethrombin 1 fraction, which lacks the phospholipid-binding domain of prothrombin, was used as immunogen to establish monoclonal APTs with phosphatidylserine-independent binding activity. We established monoclonal antibody 51A6, which as we expected, had strong APT-alone activity but lower anti-PS/PT activity. Monoclonal antibody 231D had minor APT-alone activity as compared with the 51A6 monoclonal antibody. The presence of calcium did not affect APT-alone activity in either of these monoclonal antibodies. The affinity constant (K_a) of 51A6 to prothrombin, as determined by liquid-phase inhibition ELISA, was $5.49 \times 10^{-9} M$ (data not shown), suggesting that 51A6 had moderate or strong affinity to the prothrombin molecule. The binding affinity of 231D to the PS/PT complex may be comparable to that of 51A6 according to the results of the anti-PS/PT ELISA.

Competitive ELISA revealed that 231D partially inhibited the binding to the PS/PT complex of the autoimmune anti-PS/PT antibody derived from patients with APS, implying that 231D shared the epitope(s) on phosphatidylserine-bound prothrombin with autoimmune anti-PS/PT. In contrast, 51A6 did not display any interaction in the binding between autoimmune anti-PS/PT and the PS/PT complex; thus, the 51A6 epitope on prothrombin is independent of those of autoimmune anti-PS/PT antibody.

LAC activity of monoclonal APTs alone has previously been reported (25). However, our data showed that the 231D monoclonal antibody had a stronger inhibitory effect in the APTT test than did the 51A6 monoclonal antibody, suggesting that 231D represents immunologic and hematologic properties of autoimmune anti-PS/PT antibody found in patients with APS.

Prothrombin is a single-chain glycoprotein composed of 3 structural regions as follows: fragment 1, which contains the Gla domain and kringle 1 domain, fragment 2, which mainly contains the kringle 2 domain, and a serine protease precursor domain (26,27). Prothrombin is activated and cleaved into α-thrombin in a membrane-dependent process that includes the actions of FXa, its cofactor FVa, and divalent calcium ions assembled into a complex on the membrane.

To investigate the direct effect of anti-PS/PT on thrombin generation in vitro, we prepared a chromogenic thrombin generation assay, and the effect of the 231D monoclonal antibody was explored in the presence of different concentrations of FVa and FXa. In the presence of a low concentration of FVa, 231D increased thrombin generation in a dose-dependent manner. In contrast, when a high concentration of FVa was added, 231D diminished thrombin generation. In the second set of experiments, a high concentration of FXa was found to enhance the effect of 231D in the presence of a constant concentration of FVa. When FXa was added at a high concentration, the relative FVa concentration was low, resulting in increased thrombin generation by 231D. Taken together, the balance of FVa/FXa was the determinant of the behavior of 231D with regard to the generation of thrombin, antithrombin, or prothrombin.

In the presence of sufficient amounts of FVa, the 231D monoclonal antibody decreased thrombin generation, and the 51A6 monoclonal antibody showed a similar effect, although its potential was lower. A previous study has also shown inhibitory effects of APTs on thrombin generation. Church et al (28) produced 5 monoclonal antibodies to prothrombin kringle 2, and 2 of them inhibited FVa-dependent prothrombin activation. In terms of the phospholipid-dependency of LAC-like activity shown by the phospholipid-neutralizing test in the LAC assay, the interpretation of the behavior of monoclonal APTs may be as follows: under in vitro conditions, the higher the amount of phospholipid the more prothrombinase and/or prothrombin are available, leading to the acceleration of thrombin generation in the presence of APTs.

The in vitro effects of the 231D monoclonal antibody on thrombin generation, on the other hand, are different according to the balance of FVa and FXa. Zhao et al (29) generated and characterized a human monoclonal antiprothrombin antibody with strong LAC activity that enhanced prothrombin binding to phospholipid and shortened the plasma coagulation times (29). Their data provide an explanation for the LAC paradox, showing that a single, highly purified aPL can behave as LAC and can paradoxically increase coagulation in endothelial cell–based coagulation assays. Our current findings support the hypothesis stated above, showing that the mouse monoclonal anti-PS/PT antibody 231D, which carries strong LAC activity, increased thrombin generation in the absence of FVa as well as in the presence of very low concentrations of FVa. The 231D monoclonal antibody may allow prothrombin to bind more firmly to phospholipid, assembling prothrombin on the phospholipid and subsequently increasing thrombin generation. When abundant FXa is present, FXa is able to act on prothrombin to generate thrombin. This may not be the situation when large amounts of FVa are

present, since the coenzyme activity of FVa is sufficiently potent to overcome the antithrombin generation effects of 231D. The 51A6 monoclonal antibody, needing no phospholipid involvement for its binding to prothrombin, would not play a role in the augmentation of thrombin generation in this mechanism.

This is the first study to show that monoclonal antibodies against prothrombin do not exclusively have LAC-like thrombin reduction potential, but are also able to increase thrombin generation. However, the phenomena we observed are evidently not the only mechanism of thrombosis in patients with APS. Recently, great interest has arisen with regard to the binding of aPL to procoagulant cells and how this binding mediates cell activation related to the clinical manifestations of APS. Within the last few years, studies examining the mechanism of signal transduction implicated in the induction of procoagulant substances by aPL have been performed. There is now clear evidence that the p38 MAPK pathway of cell activation plays an important role in anti-β_2GPI antibody–mediated cell activation (30–32). Considering the similarities of the properties of anti-β_2GPI and APT, procoagulant cell activation may be a major event in the generation of thrombosis in APS patients who have anti-PS/PT antibody. Thrombin may serve as a trigger for cells to express phosphatidylserine on their surface via protease-activated receptors, which are present on many types of procoagulant cells, and with glycoprotein Ib–IX–V complex on the surface of platelets, leading to platelet aggregation and activation (33).

Thrombin is a key enzyme in hemostasis and is a multipotential enzyme in the coagulation/inflammation system. The direct involvement of anti-PS/PT antibody in thrombin generation may be a clue to the pluripathologic process that occurs in patients with APS (34). Although further clarification of the roles of anti-PS/PT antibodies in APS is essential, we believe that the findings of this study contribute to the understanding of the pathophysiology of thrombophilia in patients with APS.

AUTHOR CONTRIBUTIONS

Dr. Atsumi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Atsumi, Ieko, Koike.

Acquisition of data. Sakai, Atsumi, Ieko, Amengual, Furukawa, Furusaki, Bohgaki, Kataoka, Horita, Yasuda, Koike.

Analysis and interpretation of data. Sakai, Atsumi, Ikeo, Amengual, Koike.

Manuscript preparation. Sakai, Atsumi, Amengual, Koike.

Statistical analysis. Sakai, Atsumi.

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Replication of the Association Between the *C8orf13–BLK* Region and Systemic Lupus Erythematosus in a Japanese Population

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Objective. **Recent genome-wide association studies identified an association between single-nucleotide polymorphisms (SNPs) in the *C8orf13* region of *BLK*, the B lymphoid tyrosine kinase gene, with systemic lupus erythematosus (SLE) in Caucasians. The purpose of this study was to evaluate the significance of this region in the genetic background of Japanese patients with SLE.**

Methods. **Fourteen tag SNPs in the *C8orf13–BLK* region were genotyped in 327 Japanese patients with SLE and 322 healthy Japanese controls. The population-attributable risk percentage (PAR%) of rs13277113 in Japanese was compared with that in Caucasians as well as with that of other SLE susceptibility genes in Japanese.**

Results. **As in Caucasians, rs13277113A demonstrated the strongest association in Japanese ($P = 1.73 \times 10^{-6}$ for the genotype frequency, $P = 4.75 \times 10^{-7}$ for the allele frequency, odds ratio [OR] 2.44 [95% confidence interval (95% CI) 1.43–4.16]). The association in Japanese was consistent with a recessive model ($P = 2.74 \times 10^{-7}$, OR 2.27 [95% CI 1.66–3.11]). In contrast to the Caucasian population, this risk allele was the major allele in the Japanese population. Because both the risk allele frequency and the OR were higher in Japanese than in Caucasians, the PAR% of rs13277113 was estimated to be much higher in Japanese (35.4%) than in Caucasians (16.2%), and the second highest among the 6 confirmed SLE susceptibility genes in Japanese.**

Conclusion. **The association of the *C8orf13–BLK* region with SLE was replicated in a Japanese population. Contribution of this region to the genetic predisposition to SLE appeared to be greater in Japanese than in Caucasians.**

Since it became widely used in 2006, the genome-wide association study platform has disclosed a number of susceptibility genes for complex diseases. Many such susceptibility genes have been replicated in independent studies not only of the same population, but also of different populations. Two recent studies provided convincing evidence that single-nucleotide polymorphisms (SNPs) in the *C8orf13–BLK* region of chromosome 8p23.1 are significantly associated with systemic lupus erythematosus (SLE; OMIM no. #152700) in Caucasians (1,2).

The *BLK* gene encodes a B lymphoid–specific tyrosine kinase of the Src family (3). B lineage cells express several distinct Src family protein kinases, such

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as BLK, Fyn, and Lyn. These 3 are most strikingly expressed in B cells, in fact, *BLK* is the only tyrosine kinase of the Src family that is specifically expressed in B lineage cells (3). *C8orf13* encodes a ubiquitously expressed gene, the function of which remains unknown. The risk allele of the SLE-associated SNP rs13277113 has been shown to be associated with low levels of messenger RNA (mRNA) for *BLK* and high levels of mRNA for *C8orf13* (1). Thus far, the association of the *C8orf13–BLK* region has not been tested in non-Caucasian populations.

In the present study, we examined whether the association between the *C8orf13–BLK* region and SLE could be replicated in a Japanese population. In addition, we compared the contribution of this SNP to SLE in Japanese patients with that of other previously reported and replicated SLE susceptibility genes in Japanese by estimating their population-attributable risk percentage (PAR%).

PATIENTS AND METHODS

Study population. Three hundred twenty-seven Japanese patients with SLE (24 males and 303 females; mean ± SD age 41.6 ± 13.3 years) and 322 healthy control subjects (158 males and 164 females; mean ± SD age 30.6 ± 9.5 years) were studied. All patients fulfilled the American College of Rheumatology classification criteria for SLE (4). The patients and healthy controls were recruited at Tsukuba University Hospital, Juntendo University Hospital, and University of Tokyo Hospital. All patients and controls were unrelated Japanese. Genotype data for the Caucasian population were derived from our previously reported study (1). This study was reviewed and approved by the Research Ethics Committees of the University of Tsukuba, Juntendo University, and the University of Tokyo.

Preparation of genomic DNA. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp blood kit (Qiagen, Hilden, Germany) or a QuickGene-800 kit (Fujifilm, Tokyo, Japan). Whole-genome amplification was performed using a GenomiPhi DNA Amplification kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Genotyping. TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA) was used to determine the genotype of 14 tag SNPs in the *C8orf13–BLK* region. SNPs were selected based on the HapMap Phase II data for Japanese in Tokyo, Japan (JPT). From the JPT dataset, we chose SNPs with an r^2 threshold of 0.8 and minor allele frequency of ≥0.1. The genotype frequency data in Caucasian cases and controls were obtained from our previously reported study (1).

Statistical analysis. Association analyses were calculated by chi-square tests using 2 × 2 as well as 2 × 3 contingency tables. To find the best-fit model among the recessive, additive, and dominant models, a logistic regression analysis was performed for each SNP. Since under the recessive model, rs13277113 showed the lowest *P* value on logistic regression analysis, a conditional logistic regression analysis adjusted for rs13277113 (recessive model) was further conducted for each SNP to examine the effect on SLE susceptibility after controlling for rs13277113.

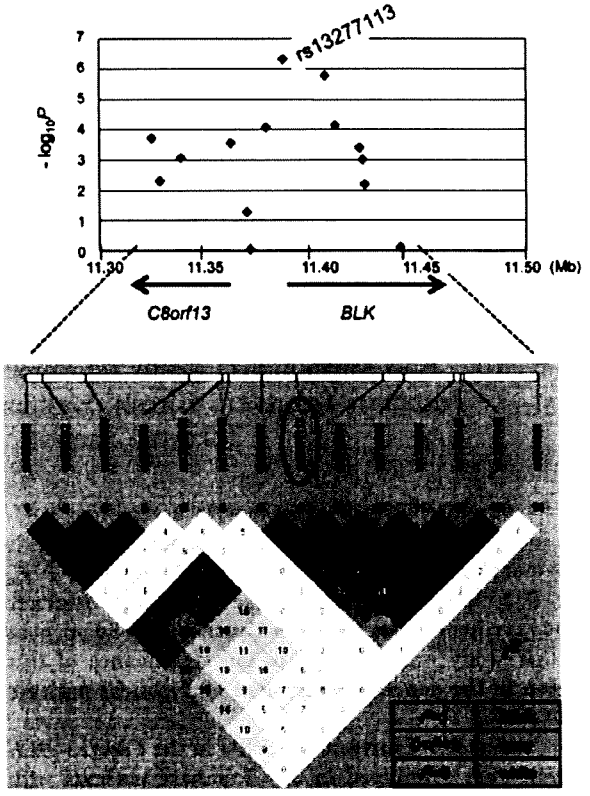

Figure 1. Linkage disequilibrium of 14 tag single-nucleotide polymorphisms (SNPs) in the *C8orf13–BLK* region in a Japanese population and allele associations with systemic lupus erythematosus. **Top,** Differences in the frequency of each of the 14 SNP alleles in the *C8orf13* and *BLK* regions. *P* values for differences in allele frequencies were calculated by chi-square test using 2 × 2 contingency tables; the $-\log_{10}$ *P* value for each SNP is shown. Arrows indicate the location and direction of transcription of *BLK* and *C8orf13*. **Bottom,** Correlation (r^2) values for each of the 14 SNPs based on data from 322 healthy Japanese control subjects. Haploview software version 4.0 (Broad Institute, Cambridge, MA; online at http://www.broadinstitute.com/haploview/haploview) was used to calculate the correlations.

The PAR% was estimated using the following formula:

$$PAR\% = Pe(RR - 1)/[Pe(RR - 1) + 1]$$

where Pe represents the risk genotype frequency in the population and RR represents relative risk of the risk genotype (5). Given the low prevalence of SLE, the Pe value can be estimated based on the genotype frequencies in healthy controls, and the RR value can be approximated by the odds ratio

Table 1. Allelic association of 14 tag SNPs in the *C8orf13–BLK* region in Japanese patients with systemic lupus erythematosus*

SNP	Chromosome position	Location	Risk allele	OR (95% CI)	*P*
rs10503423	11324491	*C8orf13* intron 2	C	1.40 (1.23–1.93)	1.93×10^{-4}
rs6984212	11328376	*C8orf13* intron 2	T	1.40 (1.11–1.77)	0.005
rs10088323	11338301	*C8orf13* intron 2	G	1.43 (1.18–1.87)	8.83×10^{-4}
rs2618431	11361874	*C8orf13* 5′ near gene	G	1.43 (1.22–1.95)	2.76×10^{-4}
rs12680762	11369436	Intergenic	T	1.41 (1.00–2.00)	0.053
rs17799348	11370931	Intergenic	T	1.01 (0.76–1.35)	0.925
rs2254891	11378539	Intergenic	C	1.66 (1.29–2.14)	8.37×10^{-5}
rs13277113	11386595	Intergenic	A	1.87 (1.47–2.40)	4.75×10^{-7}
rs2736354	11406140	*BLK* intron 1	C	1.83 (1.43–2.35)	1.67×10^{-6}
rs2736360	11410876	*BLK* intron 1	G	1.68 (1.30–2.17)	7.20×10^{-5}
rs1382566	11422250	*BLK* intron 1	G	1.57 (1.23–2.03)	3.95×10^{-4}
rs11250144	11423685	*BLK* intron 1	C	1.49 (1.17–1.88)	9.77×10^{-4}
rs12677843	11424598	*BLK* intron 1	T	1.38 (1.07–1.74)	0.006
rs2244931	11441178	*BLK* intron 3	C	1.04 (0.81–1.34)	0.762

* Odds ratios (ORs), 95% confidence intervals (95% CIs), and *P* values were calculated by chi-square test using 2 × 2 contingency tables based on allele frequencies. None of the genotypes in the controls showed significant deviation from Hardy-Weinberg equilibrium. Chromosome positions are according to the National Center for Biotechnology Information reference assembly. SNPs = single-nucleotide polymorphisms.

(OR) for each risk genotype. The PAR% for each susceptibility allele was estimated under the model (dominant or recessive) that provided the better fit, as revealed by the lower *P* value using the 2 × 2 contingency tables.

Although multiple comparisons should affect the interpretation of the statistical significance, because of the lack of a universally accepted method of correcting the *P* values for multiple SNPs in linkage disequilibrium and because our study involved the testing of a specific hypothesis, rather than being a hypothesis-generating study, we decided to present unadjusted *P* values and leave the interpretation to the reader.

RESULTS AND DISCUSSION

Fourteen tag SNPs in the *C8orf13–BLK* region were genotyped in 327 Japanese SLE patients and 322 healthy controls. Deviation from Hardy-Weinberg equilibrium was not observed for any of the SNPs in the control samples ($P = 0.134–0.955$). Figure 1 shows a linkage disequilibrium plot constructed from the Japanese control data.

Results of the case–control association analyses

Table 2. Results of logistic regression analysis of 14 SNPs for the development of systemic lupus erythematosus in Japanese, by analytical model

SNP	Correlation (r^2) with rs13277113*	Recessive model		Additive model		Dominant model	
		P†	*P* adjusted for rs13277113‡	*P*†	*P* adjusted for rs13277113‡	*P*†	*P* adjusted for rs13277113‡
rs10503423	0.326	0.002	0.445	0.0002	0.146	0.002	0.097
rs6984212	0.206	0.036	0.876	0.005	0.397	0.008	0.135
rs10088323	0.254	0.010	0.660	0.001	0.264	0.005	0.120
rs2618431	0.288	0.002	0.503	0.0004	0.223	0.005	0.162
rs12680762	0	0.042	0.064	0.053	0.081	0.008	0.799
rs17799348	0.002	0.963	0.618	0.926	0.743	0.692	0.771
rs2254891	0.763	8.85×10^{-6}	0.156	0.0002	0.461	0.078	0.858
rs13277113	NA	2.17×10^{-7}	NA	1.36×10^{-6}	NA	0.032	NA
rs2736354	0.63	1.34×10^{-7}	0.044	4.14×10^{-6}	0.211	0.157	0.808
rs2736360	0.475	1.49×10^{-5}	0.186	0.0001	0.412	0.266	0.703
rs1382566	0.434	3.27×10^{-5}	0.205	0.0006	0.657	0.616	0.283
rs11250144	0.345	0.001	0.367	0.001	0.481	0.109	0.969
rs12677843	0.313	0.004	0.580	0.007	0.854	0.245	0.662
rs2244931	0.011	0.806	0.690	0.762	0.954	0.186	0.312

* Pairwise linkage disequilibrium between rs13277113 and each of the other 13 single-nucleotide polymorphisms (SNPs) was determined by correlation (r^2) analysis of the controls. NA = not applicable.
† *P* values for each SNP under the recessive, additive, or dominant model were calculated by logistic regression analysis.
‡ *P* values were adjusted for rs13277113 under the recessive model, which provided the best fit. Under the recessive model, the association of all other SNPs lost significance, except for a marginal association of rs2736354 ($P = 0.044$).

Table 3. Estimated haplotype frequencies and results of association analyses in Japanese SLE patients and controls*

Haplotype	Allele							SLE patients	Control subjects	Permutated *P*
	rs2254891	rs13277113	rs2736354	rs2736360	rs1382566	rs11250144	rs12677843			
a	C	A	C	G	G	C	T	0.628	0.528	0.0026
b	G	G	T	A	C	G	C	0.138	0.199	0.026
c	C	A	C	G	G	G	C	0.057	0.046	0.993
d	G	G	T	G	G	C	T	0.023	0.042	0.384
g	C	G	T	A	C	G	C	0.012	0.037	0.029
e	G	G	C	G	G	C	T	0.033	0.032	1
f	C	A	T	A	C	G	C	0.028	0.031	1
h	C	A	C	G	G	C	C	0.029	0.017	0.792
i	C	A	C	G	C	G	C	0.015	0.010	0.995

* *P* values were calculated by permutation test (1,000,000 permutations) using Haploview software version 4.0 (Broad Institute, Cambridge, MA). SLE = systemic lupus erythematosus.

are shown in Table 1. Eleven of the 14 SNPs exhibited evidence of an association with SLE in our Japanese population ($P < 0.05$), among which SNP rs13277113 showed the lowest *P* value and the highest OR. Logistic regression analysis revealed that the recessive model provided the best fit for SNP rs13277113 (Table 2). SNP rs13277113 is located at the intergenic region of *BLK* and *C8orf13*, but in Japanese, it is contained in the haplotype block that includes *BLK*, but not *C8orf13*.

Most of the SNPs associated with SLE showed evidence of linkage disequilibrium with rs13277113 (Table 2 and Figure 1). However, conditional logistic regression analysis revealed that the association of all other SNPs lost statistical significance when conditioned on rs13277113 (Table 2), except for a marginal association of rs2736354 under the recessive model ($P = 0.044$).

Haplotype association analysis revealed that the major haplotype in Japanese (haplotype a) is the single risk haplotype for SLE and that it contains the rs13277113A allele (Table 3). Taken together, these results provided compelling evidence that, as in Caucasians, rs13277113A accounts for most, if not all, of the genetic effect of the *C8orf13–BLK* region in Japanese.

Table 4 shows the results of the association analysis of rs13277113 in Japanese patients with SLE as compared with that in Caucasian SLE patients. Some notable differences between the Caucasian and Japanese populations were observed. Risk allele A was the major allele in the Japanese control population, with an allele frequency of 0.652 in Japanese and 0.229 in Caucasians. In Japanese, the association was compatible with a recessive model, since the association in the heterozygotes did not reach statistical significance. On the other hand, in Caucasians, the dominant model provided a much lower *P* value, whereas under the recessive model, the OR was slightly higher. Strikingly,

Table 4. Association of rs13277113 with SLE in Japanese and Caucasian populations*

	Japanese population				Caucasian population			
	SLE patients (n = 327)	Control subjects (n = 322)	*P*	OR (95% CI)	SLE patients (n = 1,311)	Control subjects (n = 3,336)	*P*	OR (95% CI)
Genotype frequency								
A/A	207 (0.633)	139 (0.432)	1.73×10^{-6}	2.44 (1.43–4.16)	108 (0.082)	173 (0.051)	1.41×10^{-9}	1.90 (1.47–2.44)
A/G	95 (0.291)	142 (0.441)		1.10 (0.63–1.92)	551 (0.420)	1,183 (0.355)		1.41 (1.24–1.62)
G/G	25 (0.076)	41 (0.127)		Referent	652 (0.497)	1,980 (0.594)		Referent
Allele frequency								
A (vs. G)	509 (0.778)	420 (0.652)	4.75×10^{-7}	1.87 (1.47–2.39)	767 (0.293)	1,529 (0.229)	1.85×10^{-10}	1.39 (1.26–1.54)
Recessive model								
A/A (vs. A/G + G/G)	207 (0.633)	139 (0.432)	2.74×10^{-7}	2.27 (1.66–3.11)	108 (0.082)	173 (0.051)	8.55×10^{-5}	1.64 (1.28–2.10)
Dominant model								
A/A + A/G (vs. G/G)	302 (0.924)	281 (0.873)	0.032	1.76 (1.05–2.96)	659 (0.503)	1,356 (0.406)	2.60×10^{-9}	1.48 (1.30–1.68)

* *P* values, odds ratios (ORs), and 95% confidence intervals (95% CIs) were calculated by chi-square analysis using 2 × 3 (comparison of genotype frequencies) or 2 × 2 (other comparisons) contingency tables. Genotype data for the Caucasian population were obtained from our previously published study (1). SLE = systemic lupus erythematosus.

Table 5. PAR% values for the replicated systemic lupus erythematosus susceptibility genes*

Gene	Allele	Population	Model	Population frequency of risk genotype	OR of risk genotype	PAR%	Reference
C8orf13–BLK	rs13277113A	Japanese	Recessive	0.432	2.27	35.4	Present study
C8orf13–BLK	rs13277113A	Caucasian	Dominant	0.406	1.48	16.2	Present study
C8orf13–BLK	rs13277113A	Caucasian	Recessive	0.052	1.64	3.2	Present study
HLA–DRB1	DRB1*1501	Japanese	Dominant	0.124	2.97	19.6	6
FCGR2B	rs1050501C	Japanese	Recessive	0.053	2.19	5.9	8
TNFRSF1B (TNFR2)	rs60195947G	Japanese	Dominant	0.188	2.53	22.4	10
TNFSF13 (APRIL)	rs11552708G	Japanese	Dominant	0.803	2.01	44.7	12
IRF5	rs41298401C	Japanese	Recessive	0.652	1.55	26.4	14

* Population-attributable risk percentage (PAR%) values for the *C8orf13–BLK* rs13277113A allele in the Japanese and Caucasian populations were compared with the PAR% values for other systemic lupus erythematosus susceptibility alleles in the Japanese population. The model (dominant or recessive) that gave a lower *P* value by chi-square test using 2 × 2 contingency tables was selected for each allele. Because rs13277113A gave a lower *P* value under the dominant model but a slightly higher odds ratio (OR) under the recessive model in the Caucasian population, the PAR% values obtained under both models are shown.

the ORs were substantially higher in the Japanese population than in the Caucasian population.

A higher frequency of the risk genotype in the population and a higher OR result in a greater PAR%, which indicates a greater contribution of the genotype of interest in the population. Thus, the PAR% for SNP rs13277113 in the Japanese population was compared with that in the Caucasian population as well as with other SLE susceptibility genes in the Japanese population. For this purpose, we selected previously reported SLE susceptibility alleles in Japanese that have been replicated by 1 or more independent studies in Japanese or in East Asian populations, namely, *HLA–DRB1*1501* (6,7), *FCGR2B*-232Thr (rs1050501C) (8,9), *TNFRSF1B* (*TNFR2*)–196Arg (rs60195947G) (10,11), *TNFSF13* (*APRIL*)–67Gly (rs11552708G) (12,13), and *IRF5* (rs41298401C) (14,15). For each allele, the PAR% was estimated based on our previous data (6,8,10,12,14), under the dominant or recessive model that gave the better fit (lower *P* value by chi-square analysis using 2 × 2 contingency tables).

As shown in Table 5, the PAR% for rs13277113 in the Japanese population was much higher than that in the Caucasian population. This comparison may not be completely valid because the PAR% may be affected by the method of ascertainment of the patients. While all Japanese patients were ascertained at university hospitals, the Caucasian subjects consisted of multiple case–control series originally recruited for various independent studies; therefore, the method of ascertainment may vary slightly among the case–control sets (1). Nevertheless, the results strongly suggested that the contribution of rs13277113 to the development of SLE may play a greater role in Japanese than in Caucasians. In addition, the contribution of rs13277113 was the second highest among the tested susceptibility genes in the Japanese population.

This is the first replication study of the *C8orf13–BLK* SNP in a non-Caucasian population of SLE patients and controls. The findings in our Japanese population confirmed the previously reported findings in Caucasian populations (1,2) showing that the *C8orf13–BLK* region is associated with SLE. The higher frequency and OR of the risk genotype in our study population also indicated that the impact of this polymorphism is greater in Japanese SLE patients than in Caucasian SLE patients. Such information may help to identify future molecular targets for the development of new treatments.

AUTHOR CONTRIBUTIONS

Dr. Tsuchiya had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. I. Ito, Kawasaki, Behrens, Tsuchiya.

Acquisition of data. I. Ito, Kawasaki, S. Ito, Hayashi, Goto, Matsumoto, Tsutsumi, Takasaki, Hashimoto, Sumida, Tsuchiya.

Analysis and interpretation of data. I. Ito, Kawasaki, Hom, Graham, Behrens, Tsuchiya.

Manuscript preparation. I. Ito, Ohashi, Tsuchiya.

Statistical analysis. I. Ito, Hom, Ohashi.

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Prediction of efficacy of anti-TNF biologic agent, infliximab, for rheumatoid arthritis patients using a comprehensive transcriptome analysis of white blood cells

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ABSTRACT

Introduction of biologics, such as infliximab, to the therapy of rheumatoid arthritis (RA) patients has revolutionized the treatment of this disease. However, biomarkers for predicting the efficacy of the drug at an early phase of treatment for selecting real responders have not been found. We here present predictive markers based on a thorough transcriptome analysis of white blood cells from RA patients. RNA from whole blood cells of consecutive 42 patients before the first infusion was analyzed with microarrays for training studies. Samples from the subsequent 26 consecutive patients were used for a prospective study. We categorized the results into no inflammation and residual inflammation groups using the serum C-reactive protein (CRP) level at 14 weeks after the first infusion. The accuracy of prediction in our study was 65.4%.

Introduction

Rheumatoid arthritis (RA) is characterized by persistent inflammation and destruction of joints with unknown etiology. The medical management of RA has been improved from the observation that major drivers of RA inflammation are pro-inflammatory cytokines such as TNF-α and IL-6, and the strategy that blockade of these major players with biological agents should be an excellent treatment option. In the recent management of RA, tight control of the disease and early intervention with efficacious drugs such as methotrexate (MTX) and even biologics has dramatically changed the therapeutic strategy against RA, resulting in a paradigm shift. Among these biologics, three TNF blockers, infliximab, [1], etanercept [2], and adalimumab [3] have been approved and are now widely used.

There are caveats in using the biologics, however, including high cost, side effects, and individual variation of efficacy. Therefore, a reliable way for deciding whether to continue using the drug or not, as early as possible, is awaited [4,5].

In order to find biomarkers for predicting the clinical response to infliximab, we focused on a biomarker to predict diminishing inflammation, which should be tightly linked to the clinical response to TNF blockers. Thus, we utilized comprehensive transcriptome analyses of the patient's white blood cells and the serum C-reactive protein (CRP) level as an inflammation marker [6], and show the algorithm to predict the serum CRP level after infliximab treatment.

Materials and methods

Patients enrolled in this study. Forty-two and 26 consecutive patients followed at Saitama Medical Center, for retrospective and verification studies, respectively, who were resistant to standard MTX treatment, were enrolled. Written informed consent was obtained from all patients in accordance with the Helsinki protocol and with approval of the Internal Review Board of the institute.

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Clinical information. We used the serum CRP level to define the level of inflammation, 0.3 mg/dl or less as "no inflammation" (NI), and more than 0.3 mg/dl as "residual inflammation" (RI) at 14 weeks after the first treatment with infliximab infusion. According to the EULAR (European League Against Rheumatism) response criteria [7] defined based on the DAS28(CRP) scores [8] before the treatment and at week 14, the response to treatment was defined as, "good response", "moderate response", or "no response".

Total RNA extraction from blood and data processing. Blood (2.5 ml) was collected from the patient, right before the infusion of Infliximab, into PAXgene Blood RNA Kit (QIAGEN, Valencia, CA). Total RNA was extracted according to the manufacturer's protocol, and after monitoring the RNA using 260 nm absorbance, it was converted to cDNA and hybridized to Agilent whole human genome 4 × 44 K commercial oligo-DNA microarray (Agilent Technologies, Santa Clara, CA). The expression Data were normalized using Quantile algorithm (Fig. 1, [9,10]). In brief, we sorted each sample data in ascending order. Regardless of the probe name, when the rank of the probe was the same across the samples, we assigned an identical value to the probe according to the Gaussian distribution, so that all samples had the same average, variance and dynamic range, independent of day- or microarray-dependent variation. This was done to protect the low intensity probes from being filtered out.

Extracting markers and classification methods. Forty-two patients, 28 with no inflammation and 14 with residual inflammation, were subjected to training for extraction of marker genes. We used the Mann–Whitney *U*-test, calculated the *Z*-value for each probe and sorted the probes according to the significance. We then calculated the predictive values (sensitivity, specificity and accuracy) in the training set using a leave-one-out method for each probe set from the top adding recursively the next one to the set according to the rank. From the sets, the smallest number of probes and with the highest performance, in this case 10, were taken as biomarkers, and examined in the verification study with an extra set of 26 samples, calculating the accuracy, positive predictive value (PPV) and negative predictive value (NPV). We used a weighted vote method for classifying each patient. For the annotation of biomarkers, we examined the Gene Ontology terms with the top 300 Probes.

Gene Ontology analysis. In order to overview biological aspects of the biomarkers, we employed the Gene Ontology terms [11] using GO::TermFinder [12]. This tool calculates a *p*-value using

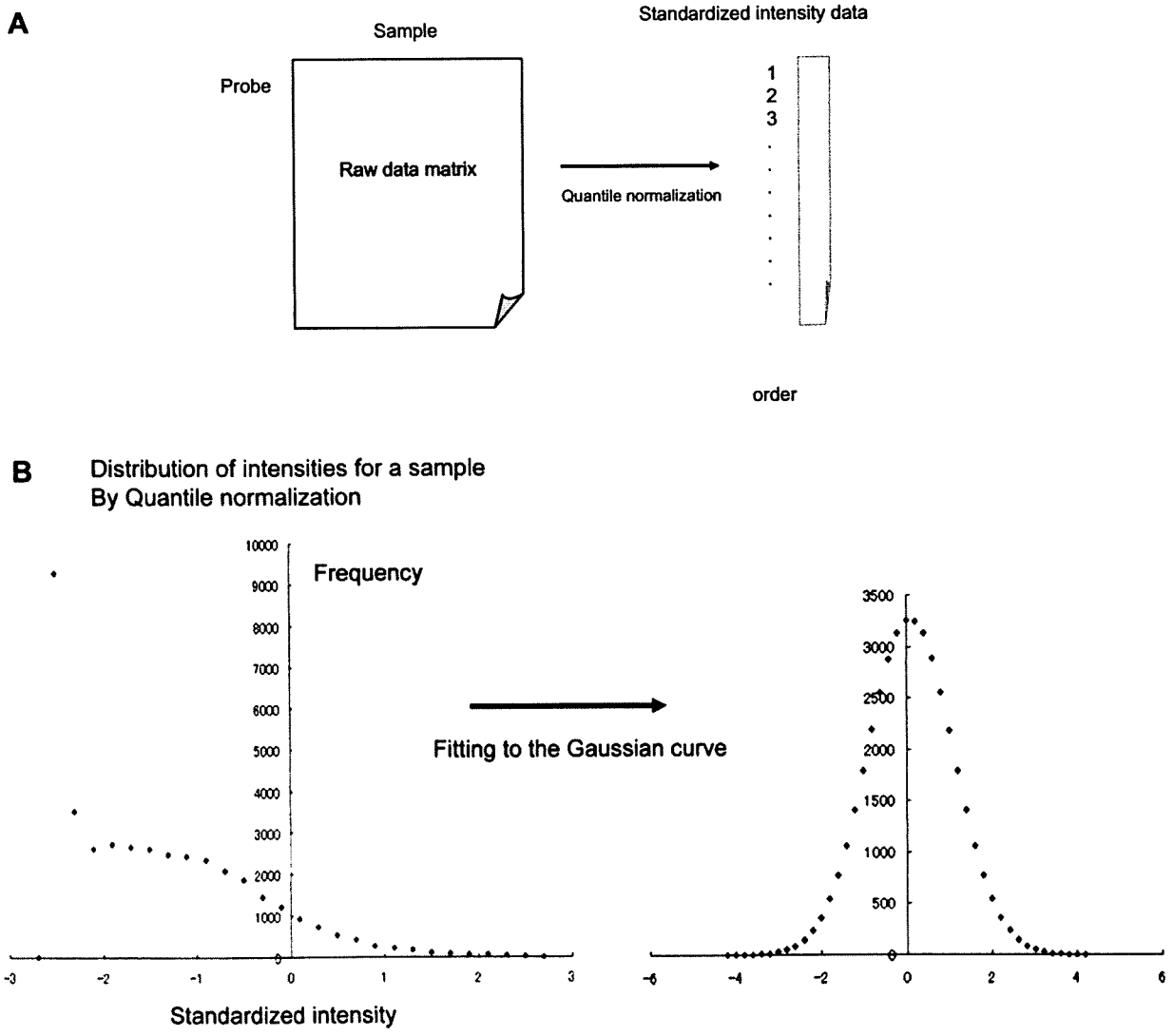

Fig. 1. Normalization method procedure. (A) All the intensity data of each sample are sorted in ascending order. Regardless of the probe name, an identical value is assigned to the probes with the same rank. (B) Fitting to the Gaussian distribution; thereby all samples have the same average, variance and dynamic range.

Table 1
Number of patients in no inflammation or residual inflammation group.

Group of patient	Number of patients in	
	No inflammation	Residual inflammation
Training set	28	14 (6)
Verification set	15	11 (8)

Forty-two patients (in Training set) and 26 patients (in Verification set) were recruited in the study.
After the first infliximab infusion, blood was taken at interval for the CRP level test. Those who had a CRP level below 0.3 mg/dl at 14 weeks were defined as having "no inflammation" (NI), and those who had a CRP level higher than 0.3 mg/dl at 14 weeks were defined as having "residual inflammation" (RI). Some of the patients in the RI group showed a consistent or transient decrease of CRP level, although above 0.3 mg/dl. The number of such patients is shown in parentheses.

the hypergenometric distribution to detect GO terms appearing in a specified list of genes, such as a marker gene set, at a frequency greater than that expected by chance.

Results

No inflammation and residual inflammation in training study

Blood was collected right before the infusion (at week 0) for RNA assay. Thereafter, blood was taken at intervals to examine the CRP level. Those who had a CRP level below 0.3 mg/dl at 14 weeks were defined as having "no inflammation" (NI), and those who had a CRP level higher than 0.3 mg/dl at 14 weeks were defined as having "residual inflammation" (RI). A retrospective study was done on 42 patients. There were 28 patients in the NI group and 14 in the RI group. We examined another 26 patients, i.e., 15 and 11 patients in the NI and RI groups, respectively, as a verification study. Table 1 summarizes the results. Supplementary Tables 1 and 2 show demographic and clinical information for the patients in retrospective and verification study, respectively. In the RI group, the CRP level of some patients at week 14 was not within the normal range (0.3 mg/dl or less), but showed a consistent or transient decrease. The number of such patients is also shown in parentheses in Table 1. The patients with "consistent decreasing" response were assigned RI1 to RI6 in Supplementary Table 1 and RI15 to RI22 in Supplementary Table 2.

RNA profiles in the training set

Total RNA was prepared from blood of the 42 patients in the retrospective study at week 0, and analyzed using Agilent microarrays. In order to select predictive markers differentiating NI from RI, we first aligned all genes (probes) along the *Z*-value obtained with Mann–Whitney *U*-test. We then calculated the predictive values for the optimal number of genes up to 300, and found that the top 10 genes on the list exhibited the highest performance using a leave-one-out method in the training set. Among these 10 probes, two were from the same gene (PSPH) and are summarized in Table 2. The specificity, sensitivity and accuracy were 96.4%, 100%, and 97.6%, respectively. A link between each probe and the Gene Ontology term is provided (see Supplementary Table 3). In Fig. 2 is visualized the expression profiles with the selected set of 10 markers across the patients using an unsupervised hierarchical method in the training set. Those having RI were essentially segregated from NI. Thus, most of the NI patients are on the left side of the graph with some exceptions, whereas all of the RI patients are on the right side.

Biological aspects of the prediction markers

Adding more markers, up to 300, to the ten in Table 2, the predictive values gradually decreased after a plateau phase. Nevertheless, the specificity, sensitivity and accuracy of the predictive values for the set of these 300 biomarkers were 78.6%, 92.9% and 83.3%, respectively. According to the Gene Ontology terms evaluated using Bonferroni corrected *p*-value, those genes were for "translation" 1.71E-08, "cellular biosynthetic process" 2.28E-05, and "oxidative phosphorylation" 9.39E-05. The implication of these genes will be dealt in detail in an accompanying paper in press [13].

Studies with verification set

Next, we adopted the 10-biomarker set for a verification study, using another 15 and 11 patients, respectively, in the NI and RI groups. The results were 65.4%, 68.8% and 60.0% for accuracy, PPV and NPV respectively (Table 3). Thus, the set of predictive markers, as obtained at week 0, was considered accurate. We next examined the predictive values for EULAR response criteria using the same 10-biomarker set. When we applied the results of prediction for the CRP criteria to EULAR response criteria, in which NI corresponds to "good response" or "moderate response" and RI to "no response", the results were 66.7%, 80.0% and 44.4% for accuracy, PPV and NPV, respectively (Table 3). The results for each patient are shown in Supplementary Table 2.

Patients showing consistent decrease or transient decrease

There is a possibility that some patients categorized as RI could, nevertheless, eventually reach NI, since their CRP level showed a decline, although not yet in the normal range (0.3 mg/dl or less). As the inflammation may be quenched gradually by the biologic, a longer time might be needed to reach NI. In contrast, some patients were "non-responders" who showed no sign of decline in CRP level during the 14-week drug administration period. Thus, we sub-divided the RI group into the consistently decreasing group, (14 patients; RI1 to RI6 in Supplementary Table 1, and RI15 to RI22 in Supplementary Table 2), and another group,

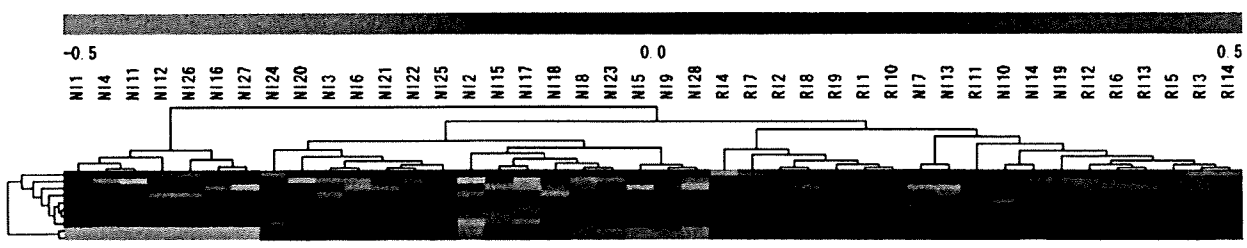

Fig. 2. Clustering of RA patients in retrospective study. RNA from 42 patients, 28 having "no inflammation" (NI) or 14 having "residual inflammation" (RI) at 0 week were analyzed using Agilent microarrays. Total RNA preparation and the data processing were done as described in Materials and Methods section. The data were analyzed using unsupervised hierarchical clustering with Euclidean distance and average linkage method using TIGR MultiExperimentViewer (MEV)-version 4.0.01.

Table 2
Ten biomarkers based on pre-treatment data.

Probe name[a]	GeneSymbol	GeneName
A_23_P251984, A_32_P78816	PSPH[b]	Phosphoserine phosphatase
A_23_P18684	CLGN	Calmegin
A_23_P132138	C21orf58	Chromosome 21 open reading frame 58
A_24_P576445	TBC1D8	TBC1 domain family, member 8 (with GRAM domain)
A_24_P76358	LOC643981	Similar to 40S ribosomal protein S3a (V-fos transformation effector protein)
A_24_P302998	ATP5I	ATP synthase, H + transporting, mitochondrial F0 complex, subunit E
A_23_P258483	ANKRD55	Ankyrin repeat domain 55
A_23_P94591	TMEM141	Transmembrane protein 141
A_32_P1144	Unknown	Unknown

Total RNA from 42 patients in the retrospective study at 0 week were analyzed with Agilent microarrays, and the data were processed as described in text. Among 300 markers that showed predictive values for differentiating "no inflammation" (NI) from "residual inflammation" (RI), the top 10 genes exhibiting the highest performance are demonstrated.

[a] Identifier used in Whole Human Genome 4 × 44 K.

[b] Two probes for one gene, PSPH, were selected in 10 markers.

non-responder, (11 patients whose CRP level was fluctuating; RI7 to RI14 in Supplementary Table 1 and RI23 to RI25 in Supplementary Table 2). We attempted to find markers to separate NI and RI consistently decreasing from RI non-responder. Because the number of patients was not large enough, we used all the data combining the 42 and 26 cases to select such markers. Using the Mann–Whitney *U*-test, we selected 300 markers (Supplementary Table 4) to separate NI plus RI consistently decreasing from RI non-responders. As can be seen in Fig. 3, these 300 markers seemed to discriminate the NI plus RI consistently decreasing group and RI non-responder group. A leave-one-out validation study, using the top 300 markers, allowed us to obtain 75.0%, 100%, and 39.3% for accuracy, PPV, and NPV, respectively. However, because the number of patients was small, we were not able to run a verification study. Further studies with more samples must be done before we can proceed to the prediction studies.

Discussion

Blood is an ideal specimen for prediction of the biologic agent, infliximab, compared to the biopsy of the cartilage or other tissues. The prediction should faithfully reflect the condition of RA and the effect of drug. Three points have been taken into consideration in this study: (1) Since the transcriptome data should be internationally interchangeable we avoided use of locally-made cDNA microarrays, and used the Agilent whole human genome 4 × 44 K product, instead; (2) to make the protocol as simple as possible, we adopted analyses of whole blood cellular RNA using PAXgene method, in which RNA of white blood cells is protected from rapid degradation, and is free from contaminating red blood cell RNA, and (3) we employed the serum CRP level, an inflammation marker, for prediction of the drug efficacy for the reason as will be discussed below. Application for prediction with serum CRP level is not a universally approved standard for RA therapy. The accepted standards for RA diagnosis are EULAR response criteria and ACR [14]. These criteria are useful for reflecting the patient's complaints and are widely appreciated in this field, but, as noted, they take into consideration the impression and complaints from patients and physicians, causing difficulty in standardized quantitative evaluation of the treatment. On the other hand, CRP, an objective measure just indicates whether the inflammation is, or is not being quenched. In addition, CRP monitoring is useful because the change of CRP is much faster than that of joint counts suffering from RA in anti-TNF biologics, particularly, in judging the response to infliximab [15]. Two categories defined by the CRP level, i.e., "residual inflammation" (RI) and "non-inflammation" (NI), are consistent with the categories defined as "no response" and "good response" or "moderate response", respectively, in the EULAR response criteria. When we applied the same analytical method using EULAR response criteria, we obtained 66.7% accuracy (Table 3). The results may look reasonable, but in reality, there are significant discrepancies depending on how we group patients. This problem will be discussed in the future.

The performance of the predictive values presented in this paper was still lower than expected, probably due to fluctuations in individual physiological conditions, and naturally, the accuracy needs to be improved. However, since the demands are high, even at this stage, it may be beneficial for both patients and doctors to adopt these results, instead of waiting for a better biomarker. The prediction, then, should come with an accuracy figure together. At the same time, we will to integrate new data every time into the database for better and improved analyses: This system may be unfamiliar in medical sciences, but it is similar to the widely used weather forecast. This trial has just started.

With the Agilent microarray platform, the terms of "cellular biosynthetic process", "oxidative phosphorylation" and "transla-

Table 3
Accuracy tests in verification study.

Clinical criterion	Results of prediction				Accuracy		
	TP[a]	FP[b]	TN[c]	FN[d]	Accuracy (%)	PPV[e] (%)	NPV[f] (%)
CRP level	11	5	6	4	65.4	68.8	60.0
EULAR response criteria	12	3	4	5	66.7	80.0	44.4

Twenty-six patients were recruited for verification study using the blood RNA at 0 week, and the 10 biomarker set in Table 2.
Two patients (NI29 and NI30) lack in EULAR response criteria data (see Supplementary Table 2). Results of prediction for each patient are also listed in Supplementary Table 2.

[a] True positive.

[b] False positive.

[c] True negative.

[d] False negative.

[e] Positive predictive value for "no inflammation" (NI) in CRP level or "good response" plus "moderate response" in EULAR response criteria.

[f] Negative predictive value for "residual inflammation" (RI) in CRP level or "no response" in EULAR response criteria.

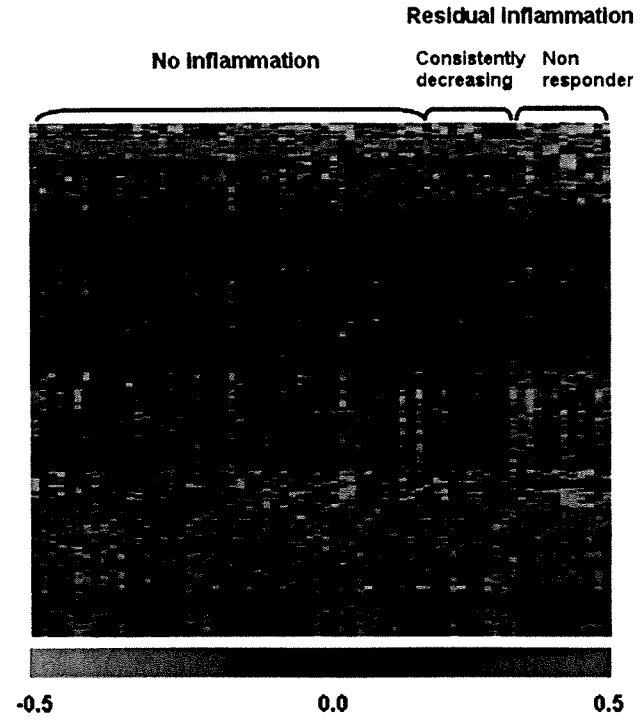

Fig. 3. Heat map of the consistently decreasing and non-responders. Heat map using genes differentially expressed between the no inflammation and RI consistently decreasing, and RI non-responders. Three hundreds genes were selected using the Mann–Whitney *U*-test.

tion" were enriched in the biomarkers extracted using the Mann–Whitney *U*-test. Thirty seven genes related to these terms are shown in Supplementary Table 5. Interestingly, 34 genes were expressed only with low intensity in those patients who were categorized in the NI group, whereas only three genes (UBA52, NME4 and TNF) were highly expressed in the same group (data not shown). High expression of TNF in patients having NI implies that the initial level of TNF is useful for determination of the efficacy of infliximab, although the biological mechanisms are yet to be solved. The low expression of the 34 genes could be reflecting the suppression of activity and/or proliferation of white blood cells. These results are in agreement with the hypothesis that the efficacy of infliximab depends partially on the trough level of the serum infliximab concentration and production of human anti-chimeric antibody (HACA) [13,16].

Lequerre et al. previously attempted to obtain biomarkers through analyses of RNA in white blood cells [4]. However, their work depended upon locally-made cDNA microarrays from a limited number of gene sets and unfortunately an evaluation using a more strict verification method was lacking. Sekiguchi et al. reported an interesting set of genes and biological aspects in TNF-blockade, but they did not obtain gene markers for prediction [5]. The present study, using 10 gene transcripts (markers), showed the usefulness of a novel system for classifying RA patients into NI and RI using whole blood samples obtained before infusion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.149.

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Gamma/Delta T Cells Are the Predominant Source of Interleukin-17 in Affected Joints in Collagen-Induced Arthritis, but Not in Rheumatoid Arthritis

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Objective. **Although interleukin-17 (IL-17)–producing γ/δ T cells were reported to play pathogenic roles in collagen-induced arthritis (CIA), their characteristics remain unknown. The aim of this study was to clarify whether γ/δ T cells or CD4+ T cells are the predominant IL-17–producing cells, and to determine what stimulates γ/δ T cells to secret IL-17 in mice with CIA. The involvement of IL-17–producing γ/δ T cells in SKG mice with autoimmune arthritis and patients with rheumatoid arthritis (RA) was also investigated.**

Methods. **IL-17–producing cells in the affected joints of mice with CIA were counted by intracellular cytokine staining during 6 distinct disease phases, and these cells were stimulated with various combinations of cytokines or specific antigens to determine the signaling requirements. Similar studies were performed using SKG mice with arthritis and patients with RA.**

Results. **Gamma/delta T cells were the predominant population in IL-17–producing cells in the swollen joints of mice with CIA, and the absolute numbers of these cells increased in parallel with disease activity. IL-17–producing γ/δ T cells expressed CC chemokine receptor 6, were maintained by IL-23 but not by type II collagen in vitro, and were induced antigen independently in vivo. Furthermore, IL-17 production by γ/δ T cells was induced by IL-1β plus IL-23 independently of T cell receptor. In contrast to what was observed in mice with CIA, IL-17–producing γ/δ T cells were nearly absent in the affected joints of SKG mice and patients with RA, and Th1 cells were predominant in the joints of patients with RA.**

Conclusion. **Gamma/delta T cells were antigen independently stimulated by inflammation at affected joints and produced enhanced amounts of IL-17 to exacerbate arthritis in mice with CIA but not in SKG mice with arthritis or patients with RA.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease that results in the destruction of cartilage and bone in joints. Collagen-induced arthritis (CIA) is a well-established murine model of this disease and shares many features with RA (1,2). Specifically, susceptibility to both CIA and RA is associated with the specific class II major histocompatibility complex allele (3,4). In addition, autoantibodies to type II collagen have been detected in the synovial fluid of patients with RA, and these autoantibodies have an aggravating effect on CIA in mice (5–7). In addition, pathogenic contributions of CD4+ T helper cells have been reported in both CIA and RA (8,9).

Interleukin-17 (IL-17) is a cytokine secreted by T cells, natural killer (NK) cells, and neutrophils (10),

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