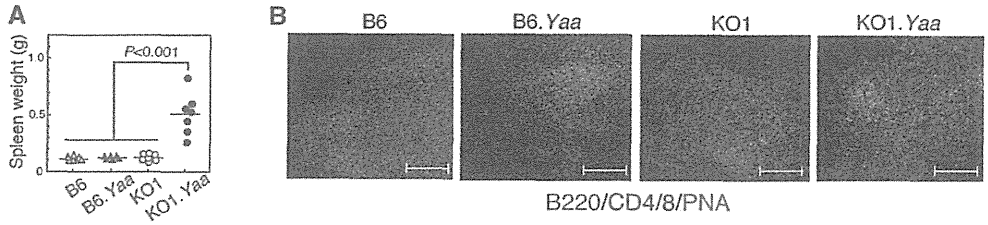

Figure 3. Splenomegaly with spontaneous GC formation in KO1.*Yaa* mice. (A) Comparison of spleen weight among B6, B6.*Yaa*, KO1, and KO1.*Yaa* mice at 4 months of age. Each symbol represents a single mouse and the bar represents the mean. Data shown are representative of three experiments performed. Statistical significance was determined by Mann–Whitney's *U* test. (B) Frozen spleen sections of 4-month-old mice were triple stained with a mixture of anti-CD4 and anti-CD8 mAbs (green), anti-B220 mAb (blue), and PNA (red) to examine the extent of GC formation. Representative results obtained from six mice in each strain are shown. Scale bar = 100 μm.

T cells was markedly increased in KO1.*Yaa* mice (Fig. 4C and Table 1). This activation of T cells may reflect the increases in the $CD4^+/CD8^+$ T-cell ratio and in the frequency of T_{FH} cells with $PD1^+ICOS^+CXCR5^+CD4^+$ phenotype (Fig. 4C and Table 1). Because the frequencies of $PD1^+ICOS^+CXCR5^+CD4^+$ T_{FH} cells in B6, B6.*Yaa*, and KO1 mice were within normal range (Table 1), the observed abnormal increase in $PD1^+ICOS^+CXCR5^+CD4^+$ T_{FH} cells in KO1.*Yaa* mice with overt SLE was thought to be due to the combined effect of the FcγRIIB-deficiency, *Sle16* locus, and *Yaa* mutation. Table 1 also shows that the frequency of $CD11b^+$ monocyte/macrophage population was significantly increased in KO1.*Yaa* mice with a comparable level observed in BXSB male mice [5].

Cytokine profile in spleen from KO1 and KO1.*Yaa* mice

To examine the difference in in vivo cytokine expression levels associated with phenotype conversion from RA to SLE, quantitative real-time PCR (qRT-PCR) analysis was performed to compare mRNA expression levels of notable cytokines in spleen between KO1 and KO1.*Yaa* mice at 4 months of age (Fig. 5A). The result showed that the expression of IL-6, IL-10, and IL-21 was significantly upregulated in KO1.*Yaa* mice compared with that in KO1 mice. Among these, the increase in IL-10 expression was prominent, with more than tenfold increase in KO1.*Yaa* mice. There was no significant difference in expression levels of other cytokines such as IL-2, IL-4, IL-17, IFN-γ, TNF-α, and IFN-α between two strains of mice.

We next examined the cellular source of IL-10 and IL-21, using flow cytometric analysis of PMA/ionomycin-stimulated spleen cells from 4-month-old KO1 and KO1.*Yaa* mice. Both IL-10 and IL-21 were secreted from $CD4^+$ T cells and the frequencies of IL-10 and IL-21-secreting cells per total $CD4^+$ T cells were significantly higher in KO1.*Yaa* than those in KO1 mice (mean ± SE of KO1 versus KO1.*Yaa*; IL-10: 7.56 ± 1.25 versus 14.74 ± 0.43, $p < 0.01$, IL-21: 5.09 ± 0.22 versus 9.91 ± 0.60, $p < 0.01$) (Fig. 5B), consistent with the results of qRT-PCR analysis. PD1 and ICOS expression levels were upregulated in in vitro stimulated $CD4^+$ T cells. Most IL-10 and IL-21-secreting cells showed high PD1 expression levels; however, the ICOS expression level was broadly distributed in these cytokine-secreting cells (Fig. 5B). As shown in Figure 5C, in addition to IL-10 and IL-21 single producers, the significant frequency of $CD4^+$ T cells secreted both cytokines.

Table 1. Subpopulations of splenocytes in KO1, KO1.*Yaa*, B6, and B6.*Yaa* mice at 4 months of age[a)]

	B6	B6.*Yaa*	KO1	KO1.*Yaa*
$B220^+$ B/total cells	50.5 ± 3.3	54.6 ± 3.4	53.5 ± 3.2	41.2 ± 5.7
$CD21^+CD23^-$ MZ B/total B	9.8 ± 0.8	2.7 ± 0.3[b)]	11.3 ± 1.2	2.3 ± 0.4[b)]
$CD69^+B220^+$ B/total B	2.7 ± 0.7	6.1 ± 1.8	2.3 ± 0.7	15.4 ± 2.8[c)]
PNA^+B220^+ B/total B	2.4 ± 0.2	2.4 ± 0.6	1.2 ± 0.1	8.1 ± 0.8[c)]
$CD138^+$ plasma/total cells	0.5 ± 0.0	1.0 ± 0.5	0.4 ± 0.1	2.6 ± 0.7[c)]
$CD3^+$ T/total cells	32.2 ± 1.6	27.9 ± 1.5	28.3 ± 2.1	18.8 ± 1.6[c)]
$CD69^+CD4^+$ T/total T	14.3 ± 2.1	19.3 ± 2.2	14.1 ± 1.3	44.6 ± 3.9[c)]
$CD4^+/CD8^+$ ratio	1.4 ± 0.1	1.6 ± 0.2	1.1 ± 0.1	6.1 ± 2.4[c)]
$CD25^+FoxP3^+CD4^+$ T/total T	18.0 ± 1.3	17.0 ± 1.3	16.0 ± 1.1	18.2 ± 0.2
$PD1^+ICOS^+CD4^+$ T/total T	2.6 ± 0.7	3.7 ± 1.2	2.1 ± 0.7	32.3 ± 3.4[c)]
$CXCR5^+PD1^+CD4^+$ T/total T	3.4 ± 0.8	3.7 ± 1.3	2.1 ± 0.7	13.2 ± 3.2[c)]
$CD11b^+$ cells/total cells	4.4 ± 0.5	4.7 ± 0.1	5.1 ± 0.2	13.3 ± 0.1[c)]

[a)] Results were obtained from six mice in each strain, and are shown as mean and SE.
[b)] The value is significantly different from B6 mice or KO1 mice ($p < 0.005$, Student's *t*-test).
[c)] The value is significantly different from other strains of mice ($p < 0.05$, Student's *t*-test).

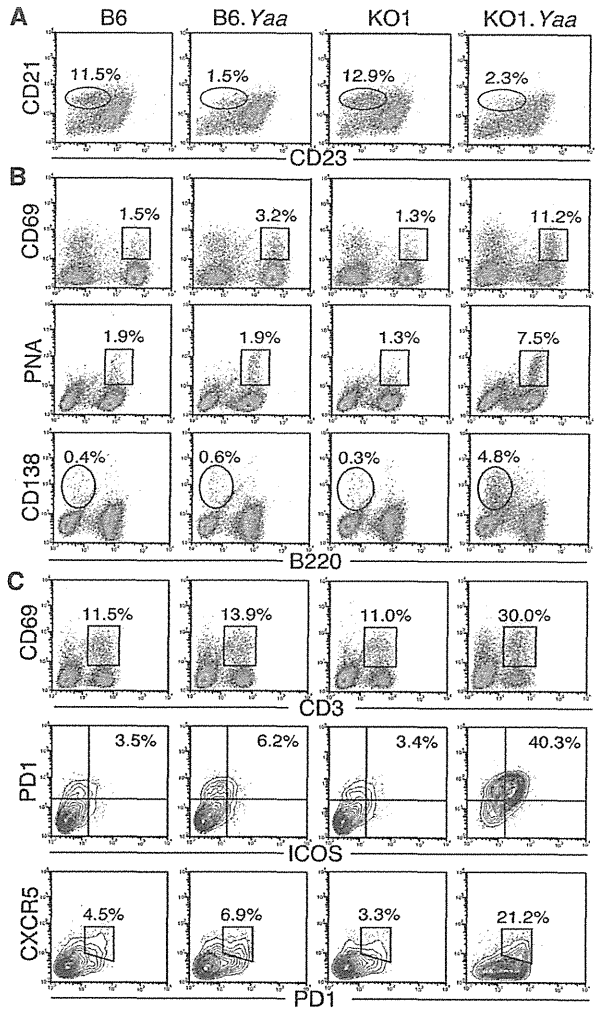

Figure 4. Comparisons of cell surface phenotypes of splenic lymphocytes among B6, B6.*Yaa*, KO1, and KO1.*Yaa* mice at 4 months of age, using flow cytometry. (A) Spleen cells were triple-stained with anti-B220, -CD21, and -CD23 mAbs, and CD21 and CD23 expression levels on $B220^+$ B cells were examined. The frequency of $CD21^+CD23^-$ marginal zone (MZ) B cells is shown. (B) Activation/maturation status of B cells. Spleen cells were stained with anti-CD69, -CD138, -B220 mAbs, and PNA. Frequencies of $CD69^+$ activated B cells per total B cells, PNA^+ GC B cells per total B cells, and $CD138^+$ plasma cells per total cells are shown. (C) Activation/maturation status of T cells. Spleen cells were stained with anti-CD69 and -CD3 mAbs, and the frequency of $CD69^+$ activated T cells per total $CD3^+$ T cells is shown. Cells were further stained with anti-CD4, -PD1, -ICOS, and -CXCR5 mAbs, and the frequencies of $PD1^+ICOS^+CD4^+$ and $PD1^+CXCR5^+CD4^+$ T_{FH} cells per total $CD4^+$ T cells are shown. Representative results obtained from six mice in each strain are shown.

Discussion

The current study showed that introduction of *Yaa* mutation into RA-prone KO1 mice leads to conversion of disease phenotypes from RA to SLE. RA and SLE are both classified as systemic autoimmune diseases. Since features of RA are occasionally associated with the clinical pictures of SLE [13], it has long been suggested that certain shared genetic pathways, as well as disease-specific ones, underlie the pathogenesis of both RA and SLE [14]. Our current model provided a clue to investigate this issue and suggested that, while the FcγRIIB deficiency and *Sle16* locus in KO1 genetic background confers predisposition to RA [6], an additional epistatic effect of *Yaa* mutation induces conversion of the disease phenotype from RA to SLE.

It has been shown that an etiology of *Yaa*-mediated B-cell activation is the duplication of the *Tlr7* gene [9, 15, 16]. The ligand for TLR7 is single-stranded RNA, thus suggesting that overexpression of TLR7 activates B cells by RNA-containing autoantigens, resulting in RNA-associated lupus autoantibody production. However, in the present study, *Yaa*-mediated disease phenotype conversion from RA to SLE was not explained by the shift of autoantibody specificity, and rather *Yaa*-mediated B-cell activation seems to be polyclonal in KO1.*Yaa* mice. This polyclonal B-cell activation may relate to the marked spontaneous GC formation and the T_{FH}-cell generation that developed in the spleen early in life of KO1.*Yaa* mice. The formation of GC depends on intrafollicular localization of antigen, activated B cells and T cells [17, 18]. Among subsets of $CD4^+$ T cells, T_{FH} cells are the specialized subset to help B cells to generate affinity-matured antibodies [17]. In addition to the B-cell help by T_{FH} cells in GC reaction, it has been shown that the relationship between B cells and T_{FH} cells is a reciprocal dependency, and that the cognate interaction with activated B cells is required for the maintenance of $PD\text{-}1^+ICOS^+CXCR5^+$ T_{FH} cells [19]. This is consistent with the present study, in which the combined effect of FcγRIIB-deficiency, *Sle16* locus, and *Yaa* mutation accelerated not only spontaneous PNA^+ B-cell generation and GC formation but also T_{FH}-cell generation in KO1.*Yaa* mice. As this vicious cycle of activated B cells and T_{FH} cells promotes polyclonal B-cell activation, KO1.*Yaa* mice showed the marked increase in serum levels of both lupus-related and RA-related autoantibodies.

Anti-CCP antibodies are currently considered to be the most specific autoantibodies for RA patients, although some patients with SLE and Sjögren's syndrome were found to have these autoantibodies [20]. Anti-CCP antibodies react with citrullinated proteins, which are the product of posttranslational modification. Citrullination of protein is a physiological process and is catalyzed by peptidyl arginine deiminase enzymes. Anti-CCP antibodies may thus gain the arthritogenicity when citrullinated proteins are increased, particularly in the arthritic region [20]. In mouse models, an increased serum level of anti-CCP antibodies was observed in SLE-prone and arthritis-free *bcl-2*-transgenic (NZW × B6)F1 mice [21], as in the case of KO1.*Yaa* mice in the current study. Thus, it appears that this autoantibody specificity is not exclusively associated with inflammatory joint diseases.

In KO1.*Yaa* mice, there was a significant increase in the IL-21 expression level early in life compared with that in KO1 mice. IL-21 is a potent immunoregulatory cytokine produced by NKT cells and $CD4^+$ T cells, and it has recently been shown that IL-21 is an autocrine growth factor for T_{FH} cells [17, 22]. Many cell types express the receptors for IL-21, but the level of expression on B cells is the highest and drives terminal differentiation of B cells and plasma cells [19, 22]. Intriguingly, Bubier et al. [23]

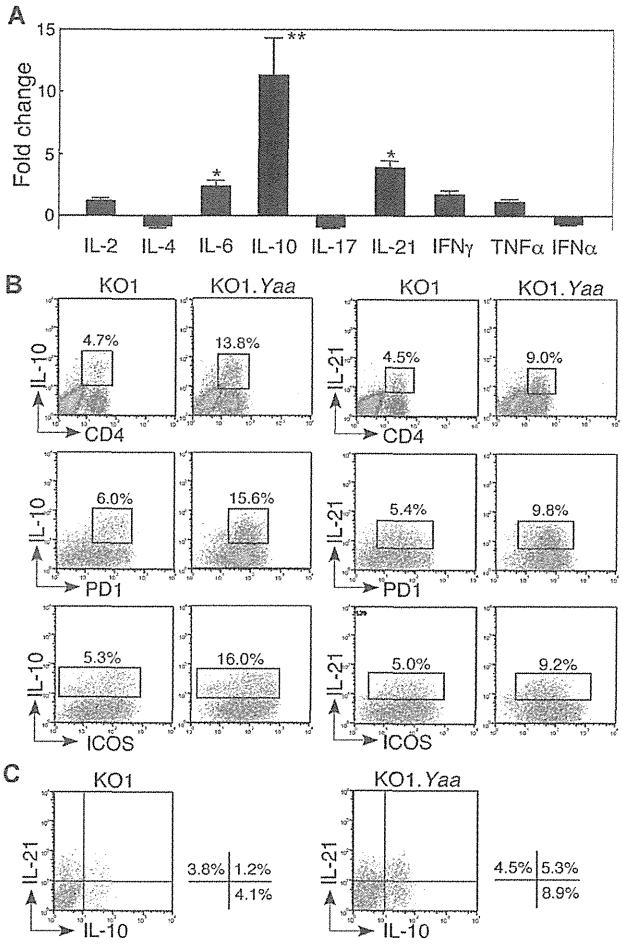

Figure 5. Comparisons of cytokine synthesis between KO1 and KO1.*Yaa* mice at 4 months of age. (A) Quantitative real-time PCR analysis of cytokine mRNA expression levels in spleen from KO1 and KO1.*Yaa* mice. Value of KO1 mice was designated as 1, and values of KO1.*Yaa* mice were evaluated as fold change compared with the values in KO1 mice. Data are shown as mean + SE of four mice for each strain and representative of three experiments performed. Statistical significance was determined by Mann–Whitney's *U* (*$p < 0.05$, **$p < 0.01$). (B) Flow cytometric analysis of IL-10 and IL-21-secreting cells in PMA/ionomycin-stimulated spleen cells from KO1 and KO1.*Yaa* mice. Frequencies of each cytokine secreting cells per total $CD4^+$ T cells, $CD4^+PD1^+$ T cells and $CD4^+ICOS^+$ T cells are shown. (C) Flow cytometric analysis of IL-10 and L-21-secreting cells, using gated $CD4^+$ T cells. Representative results obtained from six mice in each strain are shown.

reported that SLE phenotypes including autoantibody production in BXSB male mice were almost completely inhibited in the mice with the deficient IL-21 receptor. Furthermore, Rankin et al. [24] recently reported that IL-21 receptor-deficient MRL/*lpr* mice were devoid of abnormal systemic accumulation of activated B cells and T cells. These findings suggest that IL-21-mediated signals play an essential role for the pathogeneses of SLE.

It has been shown that IL-21 is a potent regulator of IL-10, since IL-10 production decreases in IL-21 receptor knockout mice, while it increases in IL-21-transgenic mice [25]. IL-10 was first described as a factor produced by Th2 cells, which inhibited the production of cytokines by Th1 cells [26]. Accumulating evidence, however, have shown that IL-10 is actually produced by many types of cells, and that, although IL-10 shows antiinflammatory properties against T cells and macrophages through inhibiting the production of inflammatory cytokines, it promotes B-cell function to induce antibody production [27, 28]. Considering these dual effects with immunosuppressive and immunostimulatory properties, IL-10 may confer different effects on the disease progression processes of RA and SLE. Indeed, the hallmark of RA is the excess production of inflammatory cytokines by T cells and macrophages at inflammatory foci, while SLE is characterized by increased production of high-affinity autoantibodies and deposition of their immune complexes in a wide variety of tissues, particularly in renal glomeruli. Consistently, there are several reports indicating the suppressive effect of IL-10 on RA [27, 29] and the promoting effect of IL-10 on lupus pathogenesis [30]. Further studies are needed to define the role of IL-10 in the conversion of disease phenotypes observed in the present study. These studies are underway in our laboratory.

Peripheral blood mononuclear cells from patients with active SLE show up-regulated expression of a group of type I IFN-induced genes [31–33]. Thus, IFN-α seems to be an important cytokine in SLE pathogenesis. In pristane-induced lupus model, the disease was shown to be associated with excess IFN-α production [34], as in the case of human SLE. However, overexpression of IFN-α is not likely to be involved in SLE pathogenesis in KO1.*Yaa* mice, since there were no differences in IFN-α expression levels between RA-prone KO1 mice and SLE-prone KO1.*Yaa* mice. Accumulating evidence shows that IL-6, IL-17, and TNF-α are important contributing cytokines to the pathogenesis of RA [35–37]. In the present studies, however, IL-6 expression levels were increased in SLE-prone KO1.*Yaa* mice compared with those in RA-prone KO1 mice, and there was no significant difference in expression levels of IL-17 and TNF-α between KO1 and KO1.*Yaa* mice. Thus, these cytokines are suggested to be unrelated to the observed phenotype conversion from RA to SLE in our model.

In conclusion, we introduced *Yaa* mutation into RA-prone KO1 strain and found that the disease phenotype converted from RA to SLE in KO1.*Yaa* mice. This phenotype conversion was likely to be due to the changes in cytokine milieus rather than the shift of autoantibody specificity from RA-related to lupus-related one. Further studies for the clarification and identification of the mechanism underlying this phenotype conversion are of paramount importance for shedding light on the mechanisms that control the development of clinically distinct systemic autoimmune diseases RA and SLE.

Materials and methods

Mice

FcγRIIB-deficient KO1 mice were generated by gene targeting in 129-derived embryonic stem cells and by backcrossing to B6 for over 12 generations [6]. The *Yaa* mutation was introduced into

KO1 mice by crossing with B6.*Yaa* mice. B6.*Yaa* mice were purchased from the Jackson Laboratory. All mice were housed under identical conditions. Experiments were performed in accordance with our institutional guidelines. Male mice were analyzed in the current study.

Incidence of arthritis

Ankle joint swelling was examined by inspection and arbitrarily scored as follows: 0, no swelling; 1, mild swelling; 2, moderated swelling; 3, severe swelling. Scores of both ankle joints are put together, and mice with scores over 2 were considered positive for arthritis.

Measurement of proteinuria

The proteinuria was monitored by biweekly testing and scored as previously descrobed [38]. Briefly, urine samples (10 μL) spotted on filter paper were air dried, fixed in 70% ethanol and stained with bromophenol blue solution. A series of standard three-fold dilution of BSA were processed as the same way, and the degree of proteinuria was assessed by visually comparing the color intensity of urine spot with that of the spot of BSA standards. Scores are as follows; 0:<37 mg/100 mL 1:≧37 mg/100 mL, 2:≧74 mg/mL, 3:≧111 mg/100 mL, 4:≧333 mg/100 mL, 5:≧1000 mg/100 mL, and 6:≧3000 mg/100 mL. Mice with urinary protein levels of four or more in repeated tests were considered as positive for proteinuria.

Histopathology and tissue immunofluorescence

Tissues fixed in 4% paraformaldehyde and embedded in paraffin were sectioned at 2 μm thickness, and tissue sections were stained with periodic acid-Schiff and hematoxylin (PAS). For immunofluorescence, tissues were embedded in Tissue-Tek OCT compound, frozen in liquid nitrogen, and sectined at 4 μm thickness. Frozen kidney sections were stained with FITC-labeled polyclonal goat anti-mouse IgG for 60 min at room temperature. For analysis of splenic tissues, frozen sections were three-color stained with Alexa 488-labeled anti-CD4 and -CD8 mAbs, Alexa 647-labeled anti-B220 mAb, and Alexa 546-labeled PNA. Antibodies and PNA were purchased from BD Pharmingen (San Diego, CA) and Vector Laboratories Inc. (Burlingame, CA), respectively. The labeling of these reagents was performed in our laboratory. Color images were obtained using laser scanning microscopy (Zeiss LSM510, Carl Zeiss Co., Ltd., Germany).

Estimation of the severity of glomerular lesion

The extent of cellular proliferation in glomerular lesion was estimated by the measurement of glomerular size. Kidney section stained by PAS was photographed under a microscope (Biozero, KEYENCE, Osaka, Japan) with ×50 magnification. Ten glomeruli in each field were randomly selected in order of size, and the size of each glomerulus was calculated using BZ-II analyzer software (KEYENCE). Mean size of 10 glomeruli was used as an indicator of histological severity of lupus nephritis in each individual mouse.

Serum levels of autoantibodies

Serum levels of IgG anti-dsDNA and -chromatin antibodies were measured using ELISA, as previously described [39]. Serum antibody levels are expressed in units, referring to a standard curve obtained by the serial dilution of a pooled serum of (NZB × NZW) F1 mice over 8 months, containing 1000 units/mL. Serum levels of IgG anti-RNP antibodies were measured by employing a commercially available kit (Alpha Diagnostic Intl. Inc., San Antonio, TX), and are expressed as relative units according to the manufacturer's instructions.

Serum levels of IgG RF and IgG anti-CCP antibodies were measured employing commercially available kits (Shibayagi Co. Ltd., Gunma, Japan and Cosmic Corporation, Tokyo, Japan, respectively), and are expressed as relative units according to the manufacturer's instructions. Serum levels of IgG anti-CII antibodies were measured using an ELISA plate precoated with bovine CII (Sigma-Aldrich, St. Louis, MO). CII-binding activities are expressed in units, referring to a standard curve obtained by serial dilution of a standard serum pool from KO1 mice hyper-immunized with CII, containing 1000 unit activities/mL.

Flow cytometric analysis

For the analysis of splenic lymphocytes, spleen cells were stained with the following reagents: FITC-conjugated anti-CD3, -CD21, -ICOS, -Foxp3, and -CD11b mAbs, Pacific blue-conjugated anti-B220, -CD4 mAbs, and PNA, PE-conjugated anti-B220, -CD138, -PD1, and -CD25 mAbs, and biotin-conjugated anti-CD69, -CD23, -CD8, and -CXCR5 mAbs, followed by streptavidin allophycocyanin. mAbs for CD25 and those for Foxp3, PD1, and ICOS were obtained from BioLegend (San Diego, CA) and eBioscience (San Diego, CA), respectively. Others were from BD PharMingen. Stained cells were four-color analyzed using a FACSAria cytometer and FlowJo software (Tree Star, Inc., Ashland, OR) with whole cell-gate excluding dead cells in forward and side scatter cytogram.

For intracellular cytokine staining of spleen cells, cells were stimulated with PMA (0.2 μg/mL)/ionomycin (2 μg/mL) in the presence of Golgi-Stop (BD Bioscience, San Jose, CA) for 5 h and stained with Pacific Blue-labeled anti-CD4 and biotin-labeled anti-PD1 or anti-ICOS mAbs followed by streptavidine allophycocyanin. Stained cells were then fixed and permeabilized using BD Cytofix/Cytoperm (BD Bioscience), followed by staining with FITC-labeled anti-IL-10, and PE-labeled anti-IL-21 mAbs. Stained cells were analyzed as above.

qRT-PCR analysis

Total RNA was isolated from spleen and first-stranded cDNA was synthesized using an oligo(dT)-primer with Superscript II First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). The cDNA product was used for each qRT-PCR sample. The data were normalized to β-actin reference. Primer pairs used were as follows: IL-2 (forward) 5′-AACCTGAAACTCCCCAGGAT-3′, (reverse) 5′-AGGGCTT GTTGAGATGATGC-3′; IL-4 (forward) 5′-CCTCACAGCAACGAA GAACA-3′, (reverse) 5′-AAGTTAAAGCATGGTGGCTCA-3′; IL-6 (forward) 5′-GACAAAGCCAGAGTCCTTCAGAGAG-3′, (reverse) 5′-CTAGGTTTGCCGAGTAGATCTC-3′; IL-10 (forward) 5′-CCAA GCCTTATCGGAAATGA-3′, (reverse) 5′-TGGCCTTGTAGACACCT TGG-3′; IL-17 (forward) 5′-TCTCTGATGCTGTTGCTGCT-3′, (reverse) 5′-GACCAGGATCTCTTGCTGGA-3′; IL-21 (forward) 5′-ATCCTGAACTTCTATCAGCTCCAC-3′, (reverse) 5′-GCATTTAGCT ATGTGCTTCTGTTTC-3′; IFNγ (forward) 5′-AAGACAATCAGGCC ATCAGC-3′, (reverse) 5′-ATCAGCAGCGACTCCTTTTC-3′; TNF-α (forward) 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′, (reverse) 5′-ACATTCGAGGCTGCTCCAGTGAATTCGG-3′; consensus IFNα (forward) 5′-ATGGCTAGRCTCTGTGCTTTCCT-3′, (reverse) 5′-AG GGCTCTCCAGAYTTCTGCTCTG-3′; β-actin (forward) 5′-AGCCAT GTACGTAGCCATCC-3′, and (reverse) 5′-CTCTCAGCTGTGGTGG TGAA-3′. The quantity was normalized using the formula of the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed using Mann–Whitney's *U* test for disease phenotypes and Student's *t*-test for flow cytometric analysis. A value of $p < 0.05$ was considered as statistically significant.

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Abbreviations: **B6**: C57BL/6 mice · **CII**: type II collagen · **CCP**: cyclic citrullinated peptide · **FcγRIIB**: IgG Fc receptor IIB · **PNA**: peanut agglutinin · **RA**: rheumatoid arthritis · **RF**: rheumatic factor · **SLE**: systemic lupus erythematosus · **qRT-PCR**: quantitative real-time PCR · ***Yaa***: Y chromosome-linked autoimmune acceleration mutation

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SUPPLEMENT

Regulatory cell subsets in the control of autoantibody production related to systemic autoimmunity

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ABSTRACT

Dysregulated autoantibody production is responsible for the severe organ damage that occurs in systemic autoimmune diseases. Immune complexes play important roles in the pathogenesis of these diseases as they initiate and maintain the inflammatory cascade, which leads to tissue destruction. Conventional therapy for autoimmune diseases suppresses immunological accelerator in the absence of knowledge of the immunological brake. Application of a physiological regulatory system could be a rational strategy to treat autoimmune diseases. Accumulating evidence has suggested that specialised subsets of B cells and T cells could control autoantibody production. A significant decrease and impaired function of regulatory B cells (Breg) was recently reported in patients with systemic lupus erythematosus and a mice model of lupus. In T cells, follicular regulatory T cells and Qa-1 restricted CD8 regulatory T cells (Treg) were identified as the populations that control follicular T helper cell-mediated antibody production. Moreover, other Treg populations might also be involved in the control of autoantibody production. Elucidating the roles of Breg and Treg in the control of antibody production might lead to the development of a new therapeutic approach to antibody-mediated autoimmune disease.

INTRODUCTION

Autoimmune diseases are fairly common disorders affecting approximately 5% of the population, predominantly women. Conventional treatment of autoimmune diseases is based mainly on the use of immunosuppressive drugs such as corticosteroids and cytotoxic reagents. These treatments reduce mortality and significantly lengthen the life expectations of patients with some diseases. However, the titres of autoantibodies are not abrogated during the course of treatment, and discontinuation of treatment often results in the relapse of disease. In systemic lupus erythematosus (SLE), anti-DNA and anti-U1-RNP antibodies often persist in spite of the immunosuppressive therapy, and the presence of these autoantibodies is associated with a poor prognosis.[1]

Autoantibodies play a pivotal role in triggering the inflammation responsible for organ damage. Immune complex deposition is readily detectable in the glomeruli of SLE. The classic hypothesis of circulating immune complex deposits inside the glomerulus was a matter of debate.[2 3] It was proposed that DNA immune complex forms in situ by binding to nucleosomes from renal cells[4] or mesangial annexin II.[5] Immune complex activates glomerular cells by the ligation of Fc receptor and the nucleic acid component of immune complex activates the renal macrophages and dendritic cells through Toll-like receptors.[3] The pathogenecity of SLE immune complex was proved by the observation that passive transfer of human SLE sera into mice expressing human FcγRIIA and FcγRIIB on neutrophils induces lupus nephritis when the mice additionally lack Mac-1.[6]

A previous study in which a proteasome inhibitor was used to deplete the plasma cell population demonstrated the importance of a continuous supply of autoantibodies for systemic autoimmunity. Bortezomib, a highly selective inhibitor of the 26S proteasome, was recently approved for use in the treatment of relapsed multiple myeloma. The pro-apoptotic effects of bortezomib in multiple myeloma are mainly due to its ability to induce the accumulation of unfolded proteins. In two lupus-prone mouse strains, NZB/W F1 and MRL/*lpr*, treatment with bortezomib depleted the number of plasma cells that are producing antibodies against double-stranded DNA; eliminated autoantibody production; ameliorated glomerulonephritis and prolonged survival. Among five bortezomib-treated mice that displayed proteinuria of greater than 100 mg/dl before treatment, four showed proteinuria of less than 100 mg/dl after treatment. These findings suggest the therapeutic potential of suppressing autoantibody production as a means of preventing organ damage in lupus.[7]

Conventional therapy for autoimmune diseases suppresses immunological accelerator in the absence of knowledge of the immunological brake. Accumulating evidence has recently shown that regulatory B cells (Breg) and regulatory T cells (Treg) control antibody production. Application of a physiological regulatory system could be a rational strategy to treat autoimmune diseases. This article will discuss the Breg and Treg-mediated suppression of autoantibody-mediated autoimmunity (figure 1).

PATHWAYS FOR AUTOANTIBODY PRODUCTION

In the steady state, most naive B cells recirculate through the lymphoid organs. On entry to the lymph nodes and spleen, B cells promptly migrate to the B-cell follicle. When B cells bind to an antigen, they become activated and express the

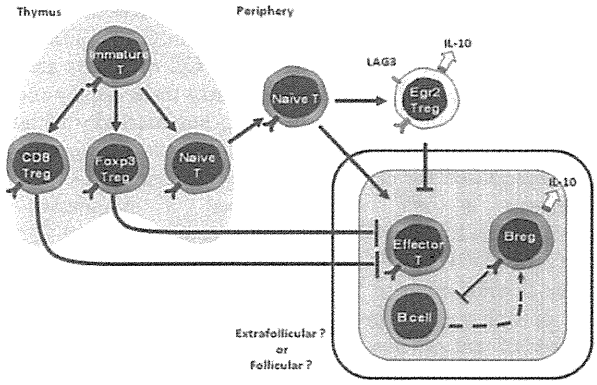

Figure 1 Schematic view of regulatory cell subsets in the control of antibody production. Breg, Foxp3$^+$Treg, CD8$^+$Treg and Egr2$^+$Treg have the capacity to control antibody production. Elucidation of the precise targets and regulatory molecules in these regulatory cell subsets would facilitate the development of a new therapeutic strategy.

chemokine receptor CCR7, which causes them to migrate to the interface of the T-cell zone and B-cell follicle. These activated B cells then differentiate along either the follicular or extrafollicular pathway.[8 9] In the follicular pathway, activated B cells migrate towards the centre of the follicle due to the downregulation of EBI2. These B cells subsequently form germinal centres (GC) and undergo somatic hypermutation and selection by interacting with CD4$^+$CXCR5$^+$PD1$^+$ follicular helper T (T_{FH}) cells. Subsequently, they exit the GC as high affinity long-lived plasma cells or memory B cells. In the extrafollicular pathway, B cells migrate to splenic bridging channels or junction zones and the borders between T-cell zones and the red pulp or extramedullary lymph node cords. This is achieved through the partial downregulation of CXCR5 and CCR7 and the upregulation of CXCR4. These migrating B cells form clusters of short-lived plasmablasts associated with dendritic cells, which aid their survival.[10] Although it is not known what kind of mechanism decides between extrafollicular plasma cell differentiation and GC migration, Paus *et al*[11] reported that antigen recognition strength regulates the choice between extrafollicular plasma cell and GC B-cell differentiation. When B cells expressing a defined B cell receptor (BCR) specificity for hen egg lysozyme were challenged with recombinant mutant hen egg lysozyme proteins engineered to bind this BCR over a 10 000-fold affinity range, B cells reactive against either high affinity or abundant epitopes were skewed towards extrafollicular plasma cell differentiation. Conversely, responding clones with weaker antigen reactivity are primarily directed to GC where they undergo affinity maturation. A number of studies suggested that both the follicular and extrafollicular pathways contribute to antibody production in murine disease models.

RESPONSE OF AUTOANTIBODY TITRES TO B-CELL DEPLETION THERAPY

Dysregulation of the follicular or extrafollicular pathway could lead to systemic autoimmune disease in mice.[12] However, it is hard to evaluate the contributions of the follicular and extrafollicular pathways to the production of disease-associated autoantibodies in humans. It is well known that the serum concentrations of some autoantibodies are correlated with disease activity, while the titres of other autoantibodies remain stable irrespective of disease status. In patients with SLE, the levels of autoantibodies that correlate with disease activity (eg, antibodies to dsDNA and nucleosomes) decrease under effective treatment, whereas those of others (such as anti-Ro and La antibodies) do not. B-cell depletion with the anti-CD20 antibody rituximab helps to distinguish between the antibodies secreted by short-lived and long-lived plasma cells because CD20 is not expressed on plasma cells.[13–15] In lupus patients, the levels of anti-nucleosome and anti-double stranded DNA antibodies are significantly decreased at 6–8 months after the administration of anti-CD20 monoclonal antibody. In contrast, the same treatment does not significantly alter their levels of anti-Ro, Sm, or RNP antibodies, or their total immunoglobulin or protective antibody titres.[14 16] This suggests that anti-nucleosome and anti-double stranded DNA antibodies are produced through extrafollicular responses, which usually generate short-lived plasma cells, while the antibodies to nucleic acid-associated antigens (Ro, Sm and RNP) that remain after rituximab therapy are derived from follicular responses, which generate long-lived plasma cells.

CONTROL OF ANTIBODY-MEDIATED AUTOIMMUNITY BY REGULATORY B CELLS

Although B cells are responsible for positive immune response, a specific and functionally unique subset of B cells, Breg, are able to regulate immune response negatively.[17] B cells are associated with the control of experimental autoimmune encephalomyelitis, chronic colitis, collagen-induced arthritis and non-obese diabetic mouse models. B cells exert their suppression via the production of interleukin (IL)-10. T2-like Breg have been identified in MRL/lpr mice.[18] Watanabe *et al*[19] reported that the transfer of splenic CD1d^{hi}CD5$^+$ B cells from wild-type NZB/W F1 mice into CD19$^{-/-}$ NZB/W F1 mice significantly prolonged their survival. Moreover, CD4$^+$CD25$^+$ Treg were significantly decreased in CD19$^{-/-}$ NZB/W F1 mice, but the transfer of wild-type CD1d^{hi}CD5$^+$ B cells induced expansion of CD4$^+$CD25$^+$ Treg in CD19$^{-/-}$ NZB/W F1 mice. Therefore, Breg may play a crucial role in the induction of Treg. Blair *et al*[20] reported that human CD19$^+$ CD24^{hi}CD38^{hi} B cells exhibit regulatory capacity. After CD40 stimulation, CD19$^+$CD24^{hi}CD38^{hi} B cells suppressed the differentiation of T-helper type 1 cells, partly via IL-10, but not transforming growth factor β (TGFβ). Interestingly, their suppressive capacity was reversed by the addition of CD80 and CD86 monoclonal antibodies. Furthermore, CD19$^+$CD24^{hi}CD38^{hi} SLE B cells isolated from the peripheral blood of SLE patients were refractory to additional CD40 stimulation, produced less IL-10 and lacked the suppressive capacity. This deficit correlated with lower levels of STAT3 phosphorylation. Altered cellular function of this population may affect effector immune responses in SLE and other autoimmune disorders. Despite phenotypic similarities, there are several differences between murine and human Breg. The suppression with murine Breg is primarily IL-10 dependent. On the other hand, although neutralisation of IL-10 in human B-cell and T-cell co-cultures completely restored tumour necrosis factor α production by T cells, IL-10 inhibition alone did not totally restore IFN-γ secretion. Moreover, while mouse Breg exhibit suppressive activity via TGFβ in experimental diabetes, suppressive activity of human CD19$^+$ CD24^{hi}CD38^{hi} B cells is not dependent on TGFβ secretion. Therefore, regulatory machinery other than IL-10 and TGFβ may be operative in human Breg.

Tim-1-mutant mice in which the mucin domain (Tim-1$^{\Delta mucin}$) was deleted showed a profound defect in IL-10 production from Breg.[21] Although Tim-1$^{\Delta mucin}$ mice do not develop frank systemic autoimmune disease, Fas-mutated

Tim-1$^{\Delta mucin}$ lpr mice developed accelerated and fulminant systemic autoimmunity with accumulation of autoantibodies to a number of lupus-associated autoantigens. When these results are taken together, expression of Tim-1 appears to be crucial in maintaining effector functions in IL-10-producing B cells. Breg functions are compromised in the presence of mutant Tim-1 that does not contain the mucin domain, and this compromise is associated with the development of autoimmunity.

Therefore, B cells themselves may control antibody-mediated autoimmunity, although the precise mechanism for suppression remains to be clarified. B-cell depletion with rituximab may affect Breg fraction and this may be one reason for the inefficiency of rituximab in SLE.

TREG SUPPRESSION OF ANTIBODY-MEDIATED AUTOIMMUNITY

Antibody suppression with CD4$^+$CD25$^+$ Foxp3$^+$ Treg

T cells are the primary sources of the help signals that promote B-cell antibody production. Therefore, suppressing antibody production at the T-cell level could be a rational therapeutic approach. Actually, several T-cell populations are able to suppress B-cell antibody production. Bystry *et al*[22] demonstrated that CD4$^+$CD25$^+$ Treg inhibit lipopolysaccharide-mediated B-cell proliferation. Although CD4$^+$CD25$^+$ Treg from human tonsil can effectively suppress T cells, they can also directly suppress B-cell response without the need to first suppress T helper cells. The direct suppression of B-cell Ig production by CD4$^+$CD25$^+$ Treg is accompanied by the inhibition of Ig class switch recombination.[23] CD4$^+$CD25$^+$ Treg from C57BL/6 mice kill antigen-presenting B cells in a cell contact-dependent manner.[24] The induction of B-cell death is not mediated by the Fas–Fas ligand pathway, but depends on the upregulation of perforin and granzymes in the CD4$^+$CD25$^+$ Treg. The CD4$^+$CD25$^+$ Treg-mediated direct suppression of B cells has also been reported in chronic systemic autoimmunity.[25] CD4$^+$CD25$^+$ Treg inhibit B-cell antibody production in in-vitro models of murine and human lupus. CD4$^+$CD25$^+$ Treg utilise granule perforin and granzyme to induce contact-dependent apoptosis in B cells. However, despite the the ability of CD4$^+$CD25$^+$ Treg from both young and old NZB/W F1 mice to suppress IgG production in B cells, autoantibody accumulation continues in these mice. In lupus-prone MRL/lpr mice with Fas mutation, CD4$^+$CD25$^+$ Treg and CD4$^+$CD25$^+$CCR2$^+$ Treg showed no therapeutic effect against glomerulonephritis and renal vasculitis, although they showed significant suppressive activity in vitro.[26] Moreover, analyses of the function and phenotypic properties of CD4$^+$CD25$^+$ Treg in SLE patients have led to conflicting results.[27] Therefore, it is not clear whether CD4$^+$CD25$^+$ Treg could be used to control autoantibody production efficiently in systemic autoimmunity.

It was recently demonstrated that the GC suppressing function of CD4$^+$CD25$^+$ Treg is limited to a subset of CD4$^+$CD25$^+$ Treg found within GC that share some characteristic of T_{FH} cells in mice and humans.[28–30] These specialised CD4$^+$CD25$^+$ Treg are termed as follicular regulatory T (T_{FR}) cells on account of their localisation and function. Acquisition of a CXCR5^{hi}PD-1^{hi} phenotype depends on interaction with B cells and SAP expression, and CXCR5 expression on Treg depends on Bcl6. CXCR5$^+$Bcl6$^+$ Treg are not detected in the thymus but can develop from CXCR5$^-$Foxp3$^+$ natural Treg precursors. T_{FR} cells can be found in GC and coexpress markers characteristic for Treg, GITR and CTLA-4 and high amounts of IL-10 messenger RNA. A deficiency of CXCR5$^+$ Treg results in enhanced GC reactions involving B cells, the affinity maturation of antibodies and plasma cell differentiation. These results indicate that the Bcl6-CXCR5 axis in CD4$^+$CD25$^+$ Treg is one regulatory mechanism by which GC responses are controlled. In addition, Foxp3-mutated scurfy mice display moderate increases in their T_{FH} populations, but markedly increased numbers of GL7$^+$CD95$^+$ GC B cells. Therefore, T_{FR} cells are more specialised for controlling the generation of GC B cells. Linterman *et al*[29] also described a population of Foxp3$^+$Blimp-1$^+$CD4$^+$ T cells that accounted for 10–25% of the CXCR5^{high}PD-1^{high}CD4$^+$ T cells found in immunised GC. In the absence of these T_{FR} cells, they observed the accumulation of non-antigen-specific B cells in GC and a decreased number of antigen-specific B cells, while the production of antigen-specific antibodies was not altered. Therefore, T_{FR} cells control GC reactions by inhibiting the selection of B cells and might suppress GC-mediated autoantibody production. However, the IL-2–STAT5 axis was recently reported to inhibit GC formation by limiting T_{FH} cell differentiation.[31] As CD4$^+$CD25$^+$ Treg consume IL-2 and cause cytokine deprivation in co-existing T cells,[32] there remains a possibility that T_{FR} cells potentially promote T_{FH} cell differentiation via IL-2 deprivation. Whether T_{FR} cells actually suppress autoantibody production remains to be evaluated and the disease induced by T_{FR} deficiency should be examined.

Lessons from IPEX syndrome

Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) is a rare X-linked recessive disorder of immune regulation due to overwhelming systemic autoimmunity.[33 34] IPEX is associated with mutations of Foxp3, which is essential for the development and functions of CD4$^+$CD25$^+$ Treg. Inflammatory enteropathy, insulin-dependent diabetes mellitus, thyroiditis, haemolytic anaemia and thrombocytopenia are frequent. However, SLE has not been diagnosed either in 'classic' IPEX babies nor in the few older children with less severe disease.[34] Notably, the production of anti-DNA antibody is rarely observed in IPEX. Although IPEX patients might develop SLE if they survive long enough, regulatory cell subsets other than Foxp3$^+$ Treg may be associated with the tolerance defect in SLE and other systemic autoimmune disease.

Antibody suppression with CD8 Treg

Recent advances in analyses of the CD8 lineage of Treg (CD8 Treg) have underscored the contribution of Qa-1-restricted CD8 Treg to the inhibition of Qa-1$^+$ T_{FH} cells. Qa-1 is a murine homologue of human leucocyte antigen E, a non-classic major histocompatibility complex (MHC) class Ib molecule encoded by the H2-T23 gene. Qa-1 binds to two receptors with opposing functions. The binding of Qa-1-peptide complexes to T-cell receptors (TCR) leads to the activation of CD8 T cells, while the binding of the Qa-1-Qdm peptide to the CD94/NKG2A receptor attenuates the activities of CD8 T cells and natural killer cells.[35] Qa-1 presents a dominant set of peptides derived from the signal sequence of classic MHC class I proteins, termed Qa-1 determinant modifiers (Qdm), as well as peptides derived from proteins associated with infectious or inflammatory responses.[36] Qa-1-binding peptides display a conserved motif consisting of a nonamer, whose termini become embedded in the antigen-binding groove, two dominant anchors and additional subdominant anchors. More than a decade ago, a subpopulation of CD8 T cells was reported to suppress T-cell help to B cells,[37] and more recent reports have demonstrated that enhanced responses to proteolipid protein self-peptide are associated with resistance of Qa-1-deficient CD4 T cells to

Qa-1-restricted CD8 T suppressor activity and increased susceptibility to experimental autoimmune encephalomyelitis.[38] Qa-1 is able to interact with both the TCR on CD8 T cells and the CD94/NKG2A receptors expressed by activated CD4 T cells. Analysis of Qa-1-restricted suppression by CD8 T cells in perforin-deficient hosts indicated a primary function for TCR-dependent rather than NKG2-dependent recognition. Moreover, expression of TCRαβ was necessary for suppressive activity, as constraints placed on TCRαβ repertoire expression resulted in loss of CD8-dependent suppressive activity. Because spontaneous autoimmunity is not observed in Qa-1-deficient mice, studies were needed to delineate fully the potential contribution of NKG2-containing receptors to suppressive activity expressed by CD8 T cells.

Kim *et al*[39] reported Qa-1 knock-in mice, B6 Qa-1 (D227K) mice, that have a Qa-1 amino acid mutation that disrupts Qa-1 binding to the TCR/CD8 complex, but the binding of Qa-1 to the inhibitory NKG2A receptor is not affected. Interestingly, B6 Qa-1 (D227K) mice displayed lupus-like systemic autoimmune disease associated with increased T_{FH} cells at approximately 6 months of age. GC formation and antibody production in response to antigen challenge are enhanced in B6 Qa-1 (D227K) mice. These phenotypes are attributed to the defect in the function of CD8 Treg, and surface phenotype analysis of CD8 Treg revealed that they express CD44, ICOSL and CXCR5. Actually, $CD44^+ICOSL^+CD8^+$ T cells have the capacity to inhibit the generation of high-affinity antibodies and Qa-1^+ T_{FH} cells. Moreover, the finding that IL-15 is required for the development and functioning of CD8 Treg facilitated the identification of three surface markers (CD44, CD122 and the class I MHC inhibitory receptor Ly49) that can be used to identify this specialised CD8 T-cell subset.[35] $Ly49^+CD8^+$ T cells efficiently suppressed CD4 T cells from wild-type mice, but not those from Qa-1 D227K mice, indicating that $Ly49^+CD8^+$ T cells display Qa-1-dependent suppressive activity. $CD44^+CD122^+Ly49^+$ CD8 T cells represent 3–5% of all CD8 T cells and account for virtually all Qa-1-restricted suppressive activity by CD8 T cells. On the other hand, the antigen specificity of CD8 Treg in T_{FH} cell suppression is not clarified because the repertoire of peptides presented by Qa-1 is substantially smaller than the repertoire of classic MHC molecules.[36] The dominant Qdm as well as peptides from HSP60, insulin, *Salmonella* GroEL and TCR Vβ chains have been identified as a limited repertoire of Qa-1 binding peptides. Therefore, CD8 Treg might control T_{FH} cells irrespective of the antigen specificity of their TCR. As CD8 Treg express CXCR5 and migrate to lymphoid follicles,[39] CD8 Treg might specifically suppress GC-mediated autoantibody production. Interestingly, the development of lupus-like disease in B6.*Yaa* mutant mice was reported to be associated with a pronounced defect in CD8 Treg activity.[40] B6.*Yaa* mice have increased numbers of T_{FH} and GC B cells at an early age, and CD8 Treg from these mice are unable to suppress WT CD4 T cells in adoptive hosts.

Possibility of antibody suppression with other Treg subsets

T_{FR} and CD8 Treg are regarded as important regulators of antibody production. Nevertheless, no Treg populations have been reported to be able to control autoantibody production and autoimmunity in an antigen-specific manner. A number of reports have described the prominent role of IL-10-producing CD4 type I Treg (Tr1 cells) cells in controlling immune responses. However, the role of Tr1 cells in the regulation of antibody production still remains unclear. In NZB/W F1 mice, high-dose tolerance to SmD_{83-119} (from D1 protein of the Smith small nuclear ribonucleoprotein) delays the production of autoantibodies and the onset of lupus, by inducing IFN-γ^+/IL-10^+ Tr1 cells.[41] On the other hand, anti-CD46-induced IL-10-secreting T cells adversely enhance antibody production by B cells.[42] These discrepancies may be due to the definition of Tr1 cells that are mainly characterised by their unique pattern of cytokine.[43 44] Several novel CD4 T-cell populations that exhibit regulatory functions have recently been reported.[45] $CD4^+CD25^-LAP^+$ T cells and $CD4^+NKG2D^+$ T cells secrete both IL-10 and TGFβ,[46 47] and $CD4^+CD25^-IL\text{-}7R^-$ T cells and $CD4^+CD25^-LAG3^+$ T cells produce large amounts of IL-10.[48 49] The capacity of these recently identified Treg populations to suppress autoantibody production should be investigated. In particular, $CD4^+CD25^-LAG3^+$ Treg, which characteristically express the anergy-associated transcription factor Egr2, might be associated with autoantibody suppression, as CD2-driven Egr2-deficient mice displayed lupus-like disease.[50] In addition, polymorphisms in the EGR2 gene are associated with human SLE susceptibility.[51] Interestingly, the adoptive transfer of $CD4^+CD25^-LAG3^+$ Treg from Fas-sufficient MRL/+ mice suppressed the progression of nephritis and autoantibody production in MRL/*lpr* lupus-prone mice (Okamura *et al*., manuscript under preparation). Therefore, $CD4^+CD25^-LAG3^+$ Treg might be useful for treating autoantibody-mediated autoimmune diseases, including SLE.[52] Although both T_{FR} cells and CD8 Treg express CXCR5 and might be associated with the suppression of GC-mediated autoantibody production, the identification of Treg populations that suppress extrafollicular responses is required for a more detailed understanding of autoantibody regulation.

CONCLUSION

The current standard treatment for autoimmune disease is non-specific immunosuppression with steroids and immunosuppressants, which frequently leads to opportunistic infections. As autoantibodies are key components in the development of autoimmune inflammation, targeting autoantibody production is a rational approach to the treatment of autoimmune diseases. Enhancing Breg and Treg functions may be one possible physiological approach to suppress autoantibody-mediated autoimmunity. Further examination of Breg, $CD4^+CD25^+$ Treg, CD8 Treg and other Treg subsets is necessary to aid the development of such treatments.

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CD247 variants and single-nucleotide polymorphisms observed in systemic lupus erythematosus patients

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Abstract

SLE is associated with a deficiency in cluster of differentiation 247 (CD247, also known as CD3 zeta chain), a component of the T-cell receptor (TCR)–CD3 complex. A comprehensive analysis showed that in more than half of SLE patients tested CD247 expression was either attenuated or absent. Recent evidence suggests that these variations in expression profiles may be due, at least in part, to polymorphisms in the *CD247* gene. Aberrant *CD247* transcript variants displaying either spliced exon 7 or short 3′-untranslated region have been detected in SLE T cells, and a recent genome-wide association study reported the existence of new *CD247* single-nucleotide polymorphisms in SLE patients. Here, we review these unique and significant features of defective CD247 observed in SLE.

Key words: systemic lupus erythematosus, T-cell receptor, signal transduction, CD247, splice variants.

Introduction

SLE is a prototype autoimmune disease characterized by an abundant production of autoantibodies and the subsequent formation of immune complexes that lead to tissue damage and clinical phenotypes such as butterfly rash and GN [1–3]. The factors of this pathogenic process are thought to be multiple and complex. For example, plasmacytoid dendritic cells activated by the innate immune system produce high levels of type I IFNs (IFN-α and IFN-β) in SLE patients. Type I IFNs affect myeloid dendritic cells and produce a number of other pro-inflammatory cytokines, resulting in the activation of immune cells such as T cells [2, 4].

T cells play a central role in both acquired immune system and immune tolerance and have been shown to be involved in various abnormalities and dysfunctions in SLE patients [4]. Functional activation of T cells is dependent on their surface expression of unique T-cell antigen receptor–cluster of differentiation 3 (TCR–CD3) complexes. TCR–CD3 complexes consist of the alpha and beta chains of TCR, associated with two epsilon, one gamma and one delta chains of CD3 and with a zeta chain [also known as CD3-zeta, TCR zeta chain or cluster of differentiation 247 (CD247)]. Here, we focus on CD247 abnormalities in SLE patients, with particular attention to gene variants and single-nucleotide polymorphisms (SNPs), and discuss how these abnormalities develop into SLE from an immunopathological perspective.

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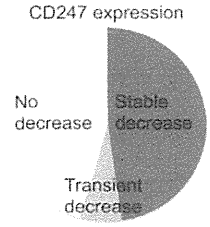
Defective CD247 expression in SLE T cells

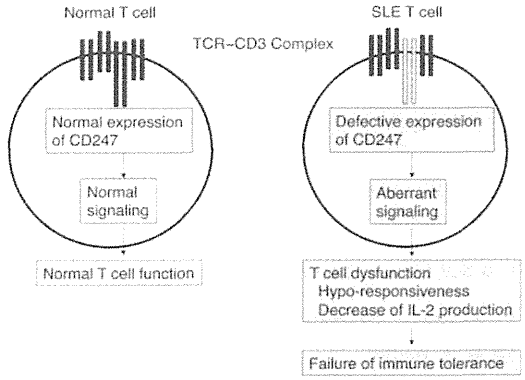
CD247 plays an important role in coupling antigen recognition to several intracellular signal transduction pathways. Our early immunoblotting analysis showed that 54.5% of SLE patients (24 out of 44) had lower (>2 S.D.) levels of CD247 protein than did healthy controls. CD247 expression, which seems to be disease-specific in the disease controls (including RA, SSc and primary SS), was not decreased. Among 44 SLE patients, CD247 expression decreased stably in 21 cases and transiently in the remaining three, suggesting the existence of several mechanisms leading to CD247 defect (Fig. 1). The relationship of CD247 expression and SLEDAI with the amount of corticosteroid administered was not significant. Furthermore, direct comparison between active and inactive phases in SLE patients showed no change in CD247 expression [5]. A decrease in TCR-initiated tyrosine phosphorylation was observed in peripheral blood T cells of SLE patients. CD247 protein expression in T-cell subpopulations, including $CD4^+$, $CD8^+$, $CD45RA^+$ (naïve phenotype) and $CD45RO^+$ (memory phenotype), was decreased. The mean CD247 fluorescence intensity in all subpopulations demonstrated a remarkably similar decrease. These results confirm the defective expression and altered tyrosine phosphorylation of CD247 in a large

FIG. 1 Defects of CD247 expression in SLE patients.



Percentage of decrease in CD247 protein less than mean ± 2 S.D. of healthy controls is shown. In 21 patients, it was stably decreased and in three patients it was transiently decreased. In total, defective CD247 expression in 54.5% of SLE patients were observed [4]. Adapted from Autoimmunity 2005;38:339–46.

FIG. 2 Defective expression of CD247 in SLE T cells.



In normal T cells, signals through the TCR–CD3 complex are transduced into internal cascades, resulting in normal T-cell function while defective expression of CD247 is observed in SLE T cells. Consequently, aberrant signalling causes T-cell dysfunction such as hyporesponsiveness and a decrease in IL-2 production, which leads to immune tolerance failure.

proportion of SLE patients, suggesting that defective expression may play an important role in SLE T-cell dysfunction [5].

In normal T cells, the TCR–CD3 complex induces intracellular signalling cascades that lead to normal T-cell function (Fig. 2), while in SLE patients diminished CD247 protein expression [6, 7] undermines the TCR–CD3 complex signalling, leading to T-cell dysfunction such as hyporesponsiveness and decreased IL-2 production, resulting in an overall immune tolerance failure.

The mechanisms responsible for this decrease in CD247 expression include low transcription activity [7], splice variant generation [6, 8, 9], increased ubiquitination [10], increased caspase-3-dependent proteolysis [10], heat stress [11], chronic pro-inflammatory cytokines exposure [12] and direct contact with activated macrophages [13]. An early CD247 northern blot analysis in T cells showed that *CD247* mRNA was undetected in three, decreased in three and normal in two out of eight SLE patients tested [6].

CD247 splice variants in SLE T cells

RNA splicing is the process by which pre-mRNA is converted into mature mRNA by removal of introns and joining of exons. Variations in splicing of the same pre-mRNA can result in the generation of splice variants that display different exon combinations.

Human *CD247* is located in chromosome 1 (1q22–q23) and consists of eight exons (Fig. 3). The existence of abnormal *CD247* transcripts was previously reported, including splice variants lacking exon 7 and variants with a short 3′-untranslated region (UTR) [5, 7, 14, 15], both of which were exclusively observed in SLE patients [16]. Other variants such as eta (exons 1–7 plus exon 9, see Fig. 3) and iota (exons 1–7 plus exon 10, not shown) are generated by alternative splicing of *CD247*.

The role of unique splice variants in defective CD247 expression

In vitro analysis of *CD247* in SLE T cells showed that mRNA instability was responsible for the lower protein expression of both the short 3′-UTR and the exon 7(–) variants. Furthermore, a T-cell transfectant model with these variants showed similar functional defects to those seen in SLE T cells [8, 15, 17–19].

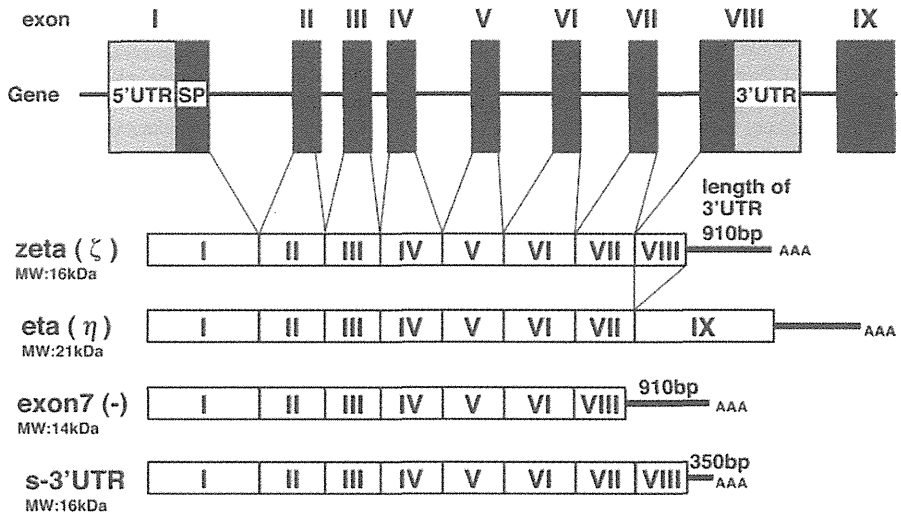
Mice bearing reduced immunoreceptor tyrosine-based activation motif (ITAM) domains in CD247 similar to those of mutated CD247 produced a substantial amount of cytokines including IFN-γ [20], which suggests that CD247 defects are linked to IFN-γ signature expression. IL-2 production from splenic T cells with all these six ITAMs of CD247 mutated was reduced in the same murine model. This is similar to human SLE T cells stimulated *in vitro*.

Although CD247 expression levels in SLE patients were found to be inversely correlated with levels of IFN-γ, both in serum and *in vitro* [21], microarray analysis of mouse transfectants carrying the human spliced variant did not detect any IFN-γ signature [22]. Further investigation on the clinical and experimental aspects of SLE will therefore be needed.

CD247 single SNPs and genome-wide association studies

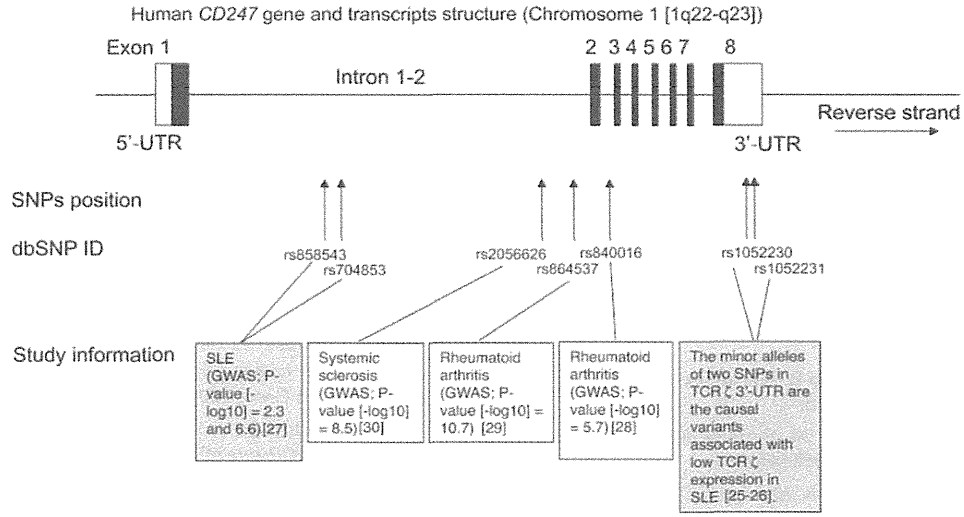
The mechanism responsible for the generation of spliced *CD247* variants in SLE patients is not yet fully understood, and conflicting observations have been reported regarding the presence or absence of mutations or deletions in the 5′-flanking region of the *CD247* gene [15, 23]. Splicing donor and acceptor sites have been reported to carry no such polymorphisms [24]. The National Center for Biotechnology Information database currently harbours

FIG. 3 Structure of known normal and spliced variants of human *CD247*.



Exon–intron organization of human *CD247* genes and their transcripts for zeta, eta and spliced variants (exon 7 deletion and short 3′-UTR) found in SLE [4]. Reproduced with permission from Autoimmunity 2005;38:339–46.

FIG. 4 SNPs of human *CD247* observed in systemic rheumatic diseases.



Schemata of human *CD247* genome and transcripts are shown with summary information of systemic rheumatic diseases related to reported SNPs (position, dbSNP ID and study information).

seven *CD247* gene SNPs that are known to be associated with systemic autoimmune diseases (http://www.ncbi.nlm.nih.gov/gene/919).

Two groups reported the existence of SNPs in the *CD247* 3′-UTR region [25, 26] (Fig. 4). They showed that the minor alleles of two of these SNPs were causal variants associated with low CD247 expression and that one-third of their mRNA was identical to that of the major alleles. The haplotype carrying the low-expression variants predisposes carriers to develop SLE [25].

CD247 was recently shown to be associated with SLE in Asian populations. A genome-wide association study in people of Chinese ethnicity identified two SNPs (rs858543 and rs704853) in the 78-kb intron 1–2 region, one of which

(rs704853) was linked to oral ulcers, haematological disorders and anti-dsDNA antibody production [27].

Two meta-analyses on RA [28, 29] and a study on systemic sclerosis [30] have reported two *CD247* SNPs located in the intron 1–2 region, one associated with RA and the other with SSc. Future analyses should focus on the functional influences of these SNPs on CD247 expression. The strength of effect of known polymorphism may not be substantial, and therefore, variation in CD247 expression must act in concert with other defects.

Conclusions

CD247 splice variants are associated in SLE with aberrant expression through either ITAM deficiency such as exon 7(–) or mRNA instability. Although the molecular mechanisms of RNA splicing are not yet fully understood, various RNA processing dysfunctions, including splicing abnormalities, were recently identified in neurological diseases [31]. We discussed here that abnormal RNA splicing processes were also found to be important in SLE pathogenesis, which suggests that more attention should be focused on new RNA-dependent diseases. Genome-wide analysis of splice variants using high-throughput sequencing and RNA processing functional assessments may improve current understanding of the topic.

Rheumatology key messages

- In SLE, defective expression of CD247 leads to T-cell dysfunction.
- *CD247* splice variants and SNPs may play a key role in SLE pathogenesis.

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Original article

Essential role of the p38 mitogen-activated protein kinase pathway in tissue factor gene expression mediated by the phosphatidylserine-dependent antiprothrombin antibody

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Abstract

Objective. The aim of this study was to investigate the effects of phosphatidylserine-dependent antiprothrombin antibody (aPS/PT) on the expression of tissue factor (TF) and the signal transduction pathway in procoagulant cells.

Methods. Peripheral blood mononuclear cells (PBMCs) from a healthy donor, murine monocyte RAW264.7 cells and human umbilical vein endothelial cells (HUVECs) were treated with either IgG fractions obtained from APS patients who were positive for aPS/PT or a murine monoclonal aPS/PT antibody, 231D, in the presence of prothrombin. The levels of TF mRNA were measured using real-time PCR. TF function, as measured by procoagulant activity, was determined with a clotting assay. 231D binding on the surface of treated cells was determined by flow cytometric analysis. Screening for phosphorylation of intracellular signalling proteins was conducted using an array assay. Phosphorylation of p38 MAPK was quantitatively analysed with ELISA, and SB203580 was used as a specific inhibitor of p38 MAPK. Specific siRNA for p38 MAPK was used for the knockdown assay.

Results. The IgG fractions from APS patients and 231D induced TF mRNA overexpression and shortening of coagulation time in cells in the presence of prothrombin. The 231D moiety induced phosphorylation of p38 MAPK after binding to the cell surface of RAW264.7 cells. SB203580 or p38 siRNA significantly hampered TF overexpression.

Conclusion. Expression of TF in procoagulant cells was induced by aPS/PT via p38MAPK phosphorylation. This phenomenon may be correlated with the thrombogenicity of APS.

Key words: antiphospholipid syndrome, antiprothrombin antibody, tissue factor, p38 MAPK, procoagulant cell activation.

Introduction

APS is a clinical condition characterized by recurrent thrombotic events and/or pregnancy morbidity associated with the persistence of aPLs. aPLs are a large and heterogeneous group of circulating immunoglobulins that appear either idiopathically or in a wide range of infectious or autoimmune diseases [1].

Traditionally aPLs are classified as aCLs, anti-beta-2-glycoprotein I (aβ_2GPI) antibodies or LA. Both aCL and aβ_2GPI are detected by ELISA, and both target the complex of β_2GPI and anionic phospholipids. These antibodies are designated β_2GPI-dependent anticardiolipin antibodies (aCL/β_2GPI) [2]. LA is detected by functional coagulation tests that require a careful and sequential series of examinations, and LA activities are indicative of the existence of heterogeneous antibodies, including aCL/β_2GPI.

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Evidence has shown that some LA activities depend on antibodies against prothrombin, which was first proposed as a possible cofactor for LA in 1959 [3]. The pathogenicity of aPT was reported from various institutes [4, 5]. Haj-Yahia *et al.* [6] reported that aPT obtained from mouse immunized with human prothrombin showed pathogenicity in an *ex vivo* model. However, association between antiprothrombin and clinical manifestation of APS is still a subject of controversy [7].

We showed that antibodies against the phosphatidylserine–prothrombin complex (aPS/PT), rather than antibodies against prothrombin alone, are closely associated with APS and LA [8], and their targeted antigen is a complex of anionic phospholipid and its binding protein, an analogue of the cardiolipin–β_2GPI complex. The sensitivity and specificity of aPS/PT for the diagnosis of APS have been assessed in a population with a variety of autoimmune disorders. It is now recognized that aPS/PT may have diagnostic potential, and they have been proposed as a candidate marker of APS and as an alternative test for LA [9–12].

In contrast to the clinical observation of a strong link between aPS/PT and thrombosis, only a few studies have demonstrated the thrombogenicity of aPS/PT. We have established a monoclonal aPS/PT, designated 231D, which specifically binds to phosphatidylserine–prothrombin complex (PS/PT) and possesses strong LA activity [13]. The concentration-dependent LA activity of the monoclonal aPS/PT and the epitope overlap reasonably represent the characteristics of autoimmune aPS/PT.

Tissue factor (TF) is the initiator of the extrinsic coagulation pathway, and we previously reported its upregulation in APS patients [14, 15]. Further, the results of our previous study and those of other studies demonstrated that monoclonal aCL/β_2GPI binds directly to procoagulant cells such as monocytes and endothelial cells (ECs), and that this binding mediates cell dysregulation, which may induce the clinical manifestations of APS [16–19]. When procoagulant cells are exposed to aCL/β_2GPI in the presence of β_2GPI, they produce thrombophilic molecules, particularly TF or adhesion molecules concomitant with activation of the p38 mitogen-activated protein kinase (MAPK) pathway [20–23]. Considering the analogy in the immunological aspects and clinical impact between aCL/β_2GPI and aPS/PT, these two populations of antibodies are likely to share in the pathophysiology of APS.

In this study we investigated the effects of aPS/PT on procoagulant cells by performing *in vitro* assays with purified IgG fractions obtained from the sera of patients with APS who were positive for aPS/PT and negative for aCL/β_2GPI, and with the monoclonal aPS/PT antibody, 231D.

Materials and methods

Monoclonal and autoimmune aPTs

Two murine monoclonal aPTs, 231D and 51A6, were previously established and characterized [13]. Briefly, the monoclonal aPS/PT antibody 231D was established as follows. BALB/c mice were intraperitoneally immunized with human prothrombin emulsified with complete or incomplete Freund's adjuvant. Spleen cells were fused with P3U1 mouse myeloma cells, and cells producing antibodies against PS/PT complex were screened using an aPS/PT ELISA, and the monoclonal antibody was sequentially purified by protein G-Sepharose affinity chromatography. 51A6, the monoclonal antibody directed against prothrombin, was established in the same manner as 231D with the exception of the immunogen used, prethrombin-1, which is a fragment of prothrombin lacking the phospholipid-binding site (Gla domain).

Both monoclonals bind strongly to the PS/PT complex, but not to phosphatidylserine alone; however, 231D has stronger binding to the PS/PT complex than 51A6. 51A6 binds to prothrombin coated on both irradiated and non-irradiated ELISA plates {antiprothrombin-alone (APT-A) activity [24]}; however, 231D shows little binding to prothrombin regardless of the plate type. 231D-spiked plasma has strong LA activity; 51A6-spiked plasma also has LA activity, but it is weaker. Binding of purified IgG from aPS/PT-positive patients with APS to the PS/PT complex is partially inhibited by 231D, but not by 51A6.

Therefore 231D has characteristics common to autoimmune aPS/PT. In contrast, 51A6 binding to prothrombin was not affected by the presence of phosphatidylserine, which is far different from the characteristics of the aPTs found in patients with APS.

IgG fractions were obtained from plasma samples of five APS patients with high titres of IgG aPS/PT in the absence of IgG aCL and aβ_2GPI using protein G-Sepharose affinity chromatography (MAbTrap-TMGII, Pharmacia). The patients included three females with a mean age of 46 (range 36–72) years, disease duration of 3–7 years and one to four past thrombotic events. IgG fractions from patients were pooled as the IgG aPS/PT fraction and frozen until use. Purified IgG fractions from plasma of three healthy individuals were prepared in the same fashion.

The study was performed in accordance with the Declaration of Helsinki and the principles of good clinical practice. Approval was obtained from the local ethics committee (Institutional Review Board of Hokkaido University Hospital), and informed consent was obtained from all subjects.

Cell isolation and preparation

Venous blood was collected from healthy donors into heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (Ficoll-Paque plus, GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). The cells were then washed with Rosewell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO BRL, Paisley, UK) containing penicillin and streptomycin, followed by centrifugation once at 400 *g* for 5 min at room temperature, and twice for 5 min at 4°C. The cells were then resuspended in RPMI-1640 and counted using the trypan blue dye exclusion method. The murine monocyte

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

Procoagulant cell treatment

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

Flow cytometry assay with IIF staining

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

RNA isolation and quantitative TaqMan real-time PCR

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

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

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

Clotting assay

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

Detection of intracellular signal protein phosphorylation

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

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

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

RNA interference

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

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

Proteins

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

Statistical analysis

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

Results

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

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

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

Procoagulant activity of PBMCs treated with aPS/PT

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

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

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

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

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

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

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

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

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

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

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

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

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