combination of ITP-associated laboratory findings, including circulating anti-GPIIb/IIIa antibody-producing B cells, reticulated platelets, and thrombopoietin [11]. The ELISPOT assay has several advantages over assays that detect platelet antigen-specific antibodies, i.e., the results are not influenced by the binding of the antibodies to platelet surfaces and only a small blood sample (<3 mL) is required. However, the anti-GPIIb/IIIa antibody response was not detectable in a small proportion (~20%) of ITP patients, even if the sensitive ELISPOT assay was used. Thus, adding a concomitant measurement of B cells producing antibodies to another major platelet autoantigen, GPIb, may increase the assay's sensitivity to the anti-platelet autoantibody response in patients with ITP. In this study, we established an ELISPOT assay for detecting anti-GPIb antibody-producing B cells and evaluated its potential usefulness for the diagnosis, disease subtyping, and assessment of the anti-platelet autoantibody profiles in patients with primary ITP and a various forms of secondary ITP.

Materials and Methods

Subjects

This study included 114 consecutive patients with primary ITP whose peripheral blood samples had been sent to an autoimmune laboratory at Keio University Hospital between April 2003 and March 2005. Eighteen patients were also included in a multicenter prospective study for verification of our preliminary diagnostic criteria for ITP [11]. The inclusion criteria were: (i) clinical diagnosis of primary ITP; (ii) thrombocytopenia (platelet count $\leq 50\times10^9$/L); (iii) no previous treatment with corticosteroids or immunosuppressants; and (iv) availability of detailed clinical information for at least one year after the diagnosis. The clinical diagnosis of ITP was made by one of the authors (YI) on the basis of clinical history, physical examination, complete blood test, and bone marrow findings if available, according to the guidelines proposed by the American Society of Hematology [12]. The final diagnosis was re-evaluated, taking into account the clinical course of the disease over at least one year, especially the therapeutic responses to corticosteroids, splenectomy, and eradication of *Helicobacter pylori* (*H. pylori*). YI was blinded to the results of the anti-GPIIb/IIIa and anti-GPIb antibody-producing B cell assays, so the clinical diagnosis of primary ITP was not influenced by these laboratory findings. Patients with primary ITP were classified as having newly diagnosed, persistent, or chronic ITP, as described previously [13].

Additional thrombocytopenic patients with underlying diseases that could potentially cause secondary ITP or non-ITP thrombocytopenia were selected from consecutive patients whose peripheral blood samples had been sent to the autoimmune laboratory during the same period, based on the definitive diagnosis of underlying diseases/conditions and platelet count $\leq 50\times10^9$/L. SLE and liver cirrhosis were diagnosed according to the published criteria [14,15]. HSCT recipients were selected based on a lack of sustained anemia or leukopenia, and no apparent cause for thrombocytopenia, such as engraftment failure, recurrence of the underlying hematologic malignancy, microangiopathy, or drugs [10]. To minimize the potential influence of procedure-related complications, we selected patients who had survived for >100 days after HSCT. Patients with aplastic anemia or myelodysplastic syndrome (MDS) were also enrolled as a non-ITP disease control. Diagnosis of aplastic anemia and MDS was based principally on bone marrow findings and cytogenetic analysis [16,17]. Thirty-two healthy individuals were also included as a control. All samples were obtained after the subjects gave their written informed consent, as approved by the ethical committee of Keio University School of Medicine (Application number 2010-031-2).

Therapeutic Response

A therapeutic response to intravenous immunoglobulin (IVIG) was defined as a platelet count $>100\times10^9$/L at one week, respectively [8], while a response to *H. pylori* eradication or corticosteroids (>0.5 mg/kg prednisolone in combination with or without IVIG) was defined as a platelet count $>100\times10^9$/L at 24 weeks, respectively [18]. We used the published definition for therapeutic response to splenectomy [19], but complete and partial responses were combined. That is, a therapeutic response was defined as a platelet count of $\geq 50\times10^9$/L for 30 days or longer after splenectomy, with or without other treatment.

H. pylori Infection

H. pylori infection was evaluated with a ^{13}C urea breath test using a UBiT tablet (Otsuka Assay, Tokyo, Japan), the detection of serum IgG anti-*H. pylori* antibodies using a commercially available kit (Kyowa Medex Company, Tokyo, Japan), and the detection of *H. pylori* antigen in stool samples using ImmunoCard® HpST® (Meridian Bioscience, Cincinnati, OH). Patients positive for the urea breath test plus at least one additional test were regarded as *H. pylori*-positive [18].

Antinuclear Antibody (ANA)

ANA was measured by indirect immunofluorescence using commercially available HEp-2 slides (MBL, Nagoya, Japan) as the substrate. A positive result was determined as a significant signal using two different cut-off levels: serum samples diluted 1:40 and 1:160.

Measurement of IgG Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells

B cells producing IgG anti-GPIIb/IIIa antibodies were measured using the ELISPOT assay as described previously [6,7]. Briefly, a polyvinylidene difluoride-bottomed 96-well microplate (Millipore, Bedford, MA) was activated by incubation with ethanol (>99.5%) at room temperature for 10 minutes. After extensive wash with phosphate-buffered saline (PBS) containing 0.5 mM $CaCl_2$ (PBS-Ca), the microplates were coated with affinity-purified human GPIIb/IIIa (purity >80%; Enzyme Research Laboratories, Swansea, UK) dissolved in PBS-Ca at a concentration of 30 μg/mL over night at 4°C. Then, the plates were washed three times with PBS-Ca, and were subsequently blocked with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS-Ca at room temperature for one hour. Peripheral blood mononuclear cells (PBMCs), isolated from heparinized peripheral blood by Lymphoprep (Fresenius Kabi Norge AS, Halden, Norway) density gradient centrifugation, were re-suspended in RPMI1640 containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA), and were pipetted into the wells (10^5/well) and cultured at 37°C with 5% CO_2 for 4 hours. After washing away the cells with PBS-Ca containing 0.05% Tween 20, the membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG (ICN/Cappel, Aurora, OH) diluted at 1:1,000 in PBS-Ca at room temperature for 2 hours, followed by wash two time each with PBS-Ca with 0.05% Tween 20 and PBS-Ca. Finally, anti-GPIIb/IIIa antibodies that bound to the membrane were visualized as spots by incubation with nitro blue tetrazolium (Sigma-Aldrich; 300 μg/mL)/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich; 60 μg/mL) in a buffer consisting of 100 mM Tris-HCl

(pH9.5), 100 mM NaCl, 50 mM $MgCl_2$ at room temperature for 20 minuts. B cells producing IgG anti-GPIb antibodies were also measured by ELISPOT assay, in which a recombinant GPIbα fragment was used instead of GPIIb/IIIa as the antigen. The recombinant GPIbα fragment, which covered the entire von Willebrand factor-binding site (residues 1 to 302), was expressed in Chinese hamster ovary cells [20]. For the anti-GPIb antibody ELISPOT assay, PBS was used instead of PBS-Ca in the entire protocol. The plates coated with bovine serum albumin in the blocking buffer in the absence of GPIIb/IIIa or GPIb were used as control for the ELISPOT assay. Each assay was conducted in 5 independent wells, and the results represented the mean of the 5 values. The frequency of circulating anti-GPIIb/IIIa or anti-GPIb antibody-producing B cells was calculated as the number per 10^5 PBMCs. The cut-off value for anti-GPIIb/IIIa antibody-producing cells was defined as 2.0 per 10^5 PBMCs [7]. The cut-off value for anti-GPIb antibody-producing cells was set at 5 standard deviations above the mean value from healthy controls.

Statistical Analysis

All continuous variables were expressed as the mean ± standard deviation (SD). Comparisons between two groups were tested for statistical significance using the Mann-Whitney test. Differences in frequency between two groups were compared using the Chi-square test or Fisher's exact test, when applicable. The correlation coefficient (r) was determined using a single-regression model.

Results

Patient Characteristics

This study enrolled a total of 226 thrombocytopenic patients. They were composed of 114 with primary ITP, 25 with SLE, 30 with liver cirrhosis, 39 with post-HSCT, and 18 non-ITP controls, including 4 with aplastic anemia and 14 with myelodysplastic syndrome. Table 1 summarizes the sex, age at examination, and platelet count of thrombocytopenia patients and healthy controls. Forty-eight patients (42%) with primary ITP were categorized as having newly diagnosed ITP, while the remaining patients had persistent or chronic ITP. The etiologies of liver cirrhosis included hepatitis B virus infection in 5, hepatitis C virus infection in 21, and alcohol in 4. Of the post-HSCT patients, 37 had received bone marrow transplantation while 2 had received peripheral blood stem cell transplantation. Compared with patients with primary ITP, patients with SLE were predominantly female ($P = 0.02$) and those with liver cirrhosis and MDS were older ($P = 0.001$ and $P = 0.03$, respectively). There was no difference in platelet count among the thrombocytopenic patient groups. The mean follow-up period in patients with primary ITP was 49±26 months.

Detection of IgG Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells

Circulating B cells producing IgG anti-GPIIb/IIIa and anti-GPIb antibodies were simultaneously measured in patients with primary ITP, various thrombocytopenic conditions, and healthy controls (Figure 1). No clear spot was detected in the control plates coated with bovine serum albumin alone. There were significantly more circulating anti-GPIIb/IIIa antibody-producing B cells in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT than in healthy controls (5.4±4.7, 6.0±6.4, 10.0±5.8, and 6.3±8.3 versus 0.3±0.4; $P<0.05$ for all comparisons). In contrast, there was no difference in anti-GPIIb/IIIa antibody-producing B cells between the non-ITP disease controls, including aplastic anemia and MDS, and healthy controls. Among ITP-related conditions, patients with liver cirrhosis had a greater frequency of anti-GPIIb/IIIa antibody-producing B cells than did those with primary ITP, SLE, or post-HSCT ($P<0.05$ for all comparisons). Similarly, there were significantly more anti-GPIb antibody-producing B cells in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT than in healthy controls (3.0±3.3, 10.5±25.6, 4.8±5.2, and 3.4±6.2 versus 0.4±0.4; $P<0.01$ for all comparisons). Again, there was no difference between the non-ITP disease controls and healthy controls. The frequency of anti-GPIb antibody-producing B cells tended to be higher in SLE patients than in those with other ITP-related conditions, but the difference was not statistically significant.

The circulating frequencies of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells were correlated with each other in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT ($P<0.0003$ for all correlations) (Figure 2). Based on the slopes of the fitted lines obtained by the single regression model, the anti-GPIIb/IIIa antibody-producing cells exceeded the anti-GPIb antibody-producing B cells in patients with primary ITP, liver cirrhosis, and post-HSCT (slope <1), whereas the anti-GPIb antibody-producing B cells predominated in SLE patients (slope >1).

Diagnostic Utility of Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells

To evaluate the diagnostic utility of the anti-GPIIb/IIIa and anti-GPIb ELISPOT assays, the results of these tests were judged as positive or negative based on being above or below a defined cut-off level. We used 2.0 per 10^5 PBMCs as the cut-off value for anti-GPIIb/IIIa antibody-producing B cells, which was determined in our previous study [7] and 2.4 per 10^5 PBMCs for circulating anti-GPIb antibody-producing B cells, which was 5 standard deviations above the mean value obtained from healthy controls. The positive frequencies of circulating anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells, and their combination in patients with primary ITP, various thrombocytopenic conditions, and healthy controls are summarized in Table 2. Anti-GPIIb/IIIa antibody-producing cells were detected in 86% of the patients with primary ITP, and in 76%, 97%, and 62% of the patients with SLE, liver cirrhosis, and post-HSCT, respectively. In contrast, the percentages of patients with a positive frequency of anti-GPIb antibody-producing B cells were lower (38–50%) than those of anti-GPIIb/IIIa antibody-producing cells. These antibody-producing cells were infrequently detected in patients with aplastic anemia or MDS. Of 16 patients with primary ITP who were negative for the anti-GPIIb/IIIa antibody-producing cells, 5 (31%) were positive for the anti-GPIb antibody-producing cells. Three (50%) out of 6 SLE patients with the negative anti-GPIIb/IIIa ELISPOT result were positive for the anti-GPIb ELISPOT assay, but none of the patients with liver cirrhosis or post-HSCT who showed the negative anti-GPIIb/IIIa ELISPOT result were positive for the anti-GPIb ELISPOT assay. When the results for anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells were combined, the positive frequency was slightly increased in patients with primary ITP and SLE, but not in those with liver cirrhosis and post-HSCT, because in the latter cases the anti-GPIb antibody-producing B cells always coexisted with anti-GPIIb/IIIa antibody-producing ones.

We then focused on the utility of anti-GPIIb/IIIa and anti-GPIb antibody-producing B cell measurement for the diagnosis of primary ITP. This analysis included 114 patients with primary ITP and 18 with non-ITP thrombocytopenia, including aplastic anemia and MDS. The anti-GPIIb/IIIa antibody-producing B cell measurement had a sensitivity of 86%, specificity of 83%, positive

Table 1. Demographic features and platelet count of subjects enrolled in this study.

	Number	Sex (% male)	Age at examination (years)	Platelet count ($\times 10^9$/L)
Primary ITP	114	40%	49.6±17.1	28.1±11.6
SLE	25	12%	43.6±14.6	28.6±13.3
Liver cirrhosis	30	53%	63.3±9.1	35.2±11.5
Post-HSCT	39	59%	37.6±10.6	31.5±11.3
Aplastic anemia	4	25%	46.3±23.9	24.5±16.2
MDS	14	57%	60.4±17.5	26.9±13.3
Healthy controls	32	50%	44.1±12.2	252.4±56.8*

ND: not determined.
*Data were derived from 16 healthy donors.
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predictive value of 98%, and negative predictive value of 50%. In contrast, the sensitivity of the anti-GPIb antibody-producing cells was only 53%, while the specificity was 89% and positive and negative predictive values were 86% and 20%, respectively. When the two tests were combined, the sensitivity was slightly improved to 90% without effectively reducing the specificity or positive predictive value of the anti-GPIIb/IIIa ELISPOT assay alone (83%, 97%, respectively). When the same analysis was performed in patients with SLE, the sensitivity was improved from 76% in case of the anti-GPIIb/IIIa ELISPOT assay alone to 88% in case of combining the two tests.

Clinical Characteristics Associated with Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells in Patients with Primary ITP

Patients with primary ITP were stratified into two groups based on the presence or absence of anti-GPIIb/IIIa or anti-GPIb antibody-producing B cells (Table 3). Newly diagnosed ITP was less common in patients with anti-GPIIb/IIIa antibody-producing B cells, than in those without. The positive anti-GPIb ELISPOT assay result was associated with a low platelet count, lack of *H. pylori* infection, and positive ANA, independent of the cut-off levels. Therapeutic responses to *H. pylori* eradication, IVIG, and splenectomy tended to be worse in patients with a positive anti-GPIb ELISPOT assay than in those without, but only the difference in the response to IVIG reached statistical significance. On the other hand, there were no differences in clinical

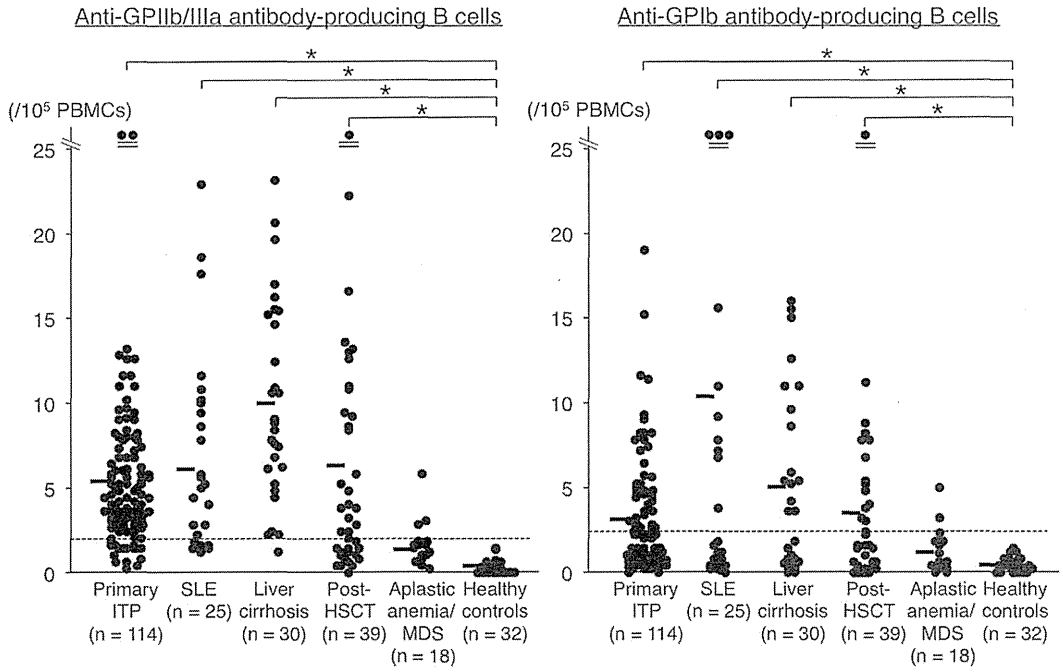

Figure 1. Anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells in the circulation of patients with various thrombocytopenic conditions and healthy controls. Cut-off values for anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells were 2.0 and 2.4 per 10^5 PBMCs, respectively. Bars indicate the mean, and asterisks indicate statistical significance ($P<0.05$).
doi:10.1371/journal.pone.0086943.g001

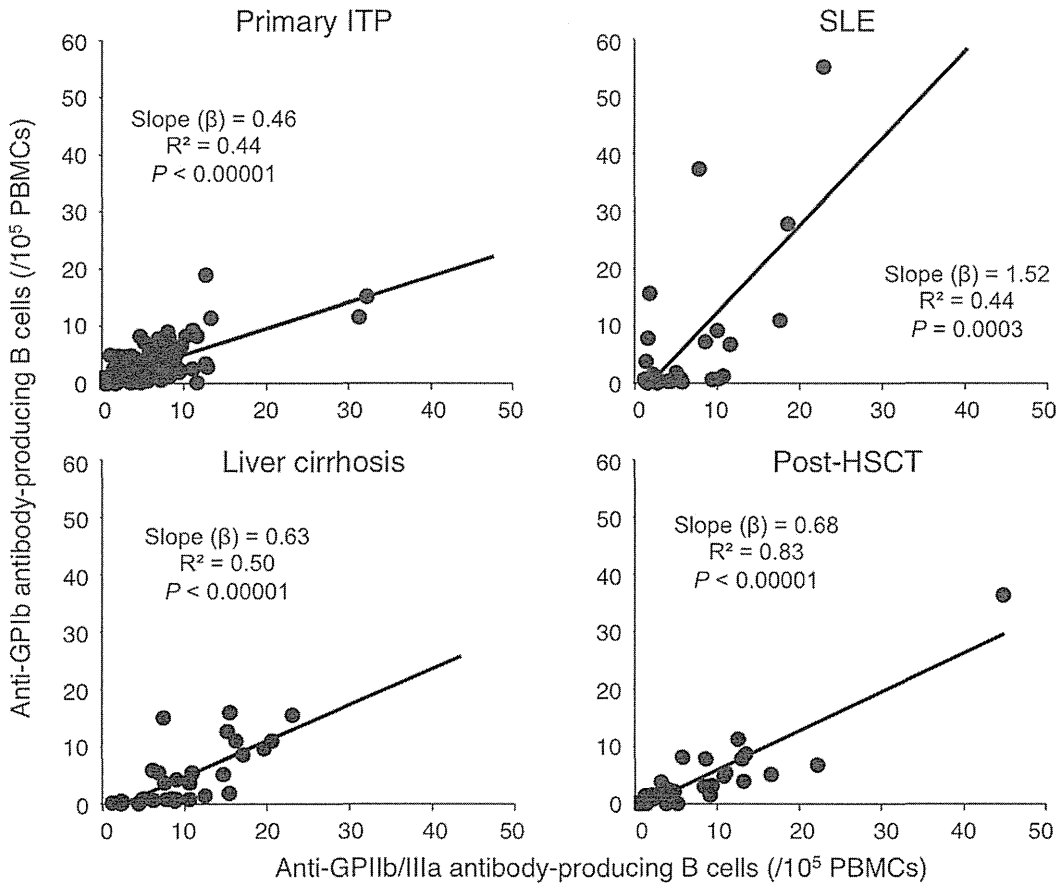

Figure 2. Correlations between circulating anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT.
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characteristics except the ITP classification between patients with and without anti-GPIIb/IIIa antibody-producing B cells.

Discussion

In this study, we successfully developed an ELISPOT assay for detecting anti-GPIb antibody-secreting B cells, by applying the principles of our previously developed anti-GPIIb/IIIa antibody-producing B cell measurement. Anti-GPIb antibody-secreting B cells were detected in the circulation of patients with primary ITP as well as conditions that potentially cause secondary ITP, but were infrequently found in patients with aplastic anemia or MDS. Thus, the anti-GPIb ELISPOT assay is useful for identifying patients with ITP, but its sensitivity was much inferior to that of the anti-GPIIb/IIIa ELISPOT assay, indicating that detection of anti-GPIb antibody-producing cells could not replace the anti-GPIIb/IIIa assessment in ITP diagnosis. In addition, concomitant measurement of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells had limited utility: a slight increase in sensitivity only for primary ITP and SLE. These findings indicate that, rather

Table 2. Positive frequencies of circulating anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells, and their combination in patients with primary ITP, various thrombocytopenic conditions, and healthy controls.

	Primary ITP (n = 114)	SLE (n = 25)	Liver cirrhosis (n = 30)	Post-HSCT(n = 39)	Aplastic anemia/ MDS (n = 18)	Healthy controls (n = 32)
Anti-GPIIb/IIIa antibody-producing B cells alone	86%	76%	97%	62%	17%	0%
Anti-GPIb antibody-producing B cells alone	43%	40%	50%	38%	11%	0%
Anti-GPIIb/IIIa antibody-producing B cells AND anti-GPIb antibody-producing B cells	38%	28%	50%	38%	11%	0%
Anti-GPIIb/IIIa antibody-producing B cells OR anti-GPIb antibody-producing B cells	90%	88%	97%	62%	17%	0%

SLE, liver cirrhosis, and post-HSCT are conditions potentially causing secondary ITP, whereas aplastic anemia and MDS are non-ITP disease controls.
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Table 3. Clinical findings in patients with primary ITP, stratified by the presence or absence of circulating anti-GPIb or anti-GPIIb/IIIa antibody-producing B cells.

	Anti-GPIIb/IIIa antibody-producing B cells			Anti-GPIb antibody-producing B cells		
	Present (n = 98)	Absent (n = 16)	*P*	Present (n = 49)	Absent (n = 65)	*P*
Sex (% female)	58%	69%	0.60	65%	55%	0.28
Age at examination (years)	50.3±17.5	45.4±14.1	0.29	50.0±17.6	49.3±16.9	0.85
Newly diagnosed ITP (%)	37%	75%	0.009	47%	38%	0.47
Platelet count (x 10^9/L)	27.5±11.5	31.7±12.1	0.19	19.8±9.4	34.4±8.8	<0.0001
H. pylori infection	28%	19%	0.66	14%	35%	0.01
Positive ANA (≥1:40)	26%	44%	0.23	51%	23%	0.002
Positive ANA (≥1:160)	17%	13%	0.83	24%	5%	0.004
Therapeutic response						
H. pylori eradication	62% (n = 26)	100% (n = 3)	0.50	33% (n = 6)	74% (n = 23)	0.16
IVIG	65% (n = 40)	56% (n = 9)	0.60	46% (n = 24)	80% (n = 25)	0.03
Corticosteroids	21% (n = 53)	17% (n = 12)	0.91	24% (n = 33)	15% (n = 33)	0.54
Splenectomy	76% (n = 37)	75% (n = 8)	0.97	64% (n = 22)	86% (n = 23)	0.14

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disappointingly, the additional measurement of anti-GPIb antibody-producing B cells on top of the anti-GPIIb/IIIa ELISPOT assay does not improve the diagnostic accuracy for patients suspected of having ITP. However, it may be worth measuring the anti-GPIb ELISPOT assay in patients who are suspected to have primary ITP or secondary ITP in association with SLE, but are negative for anti-GPIIb/IIIa antibody-producing cells, because there is a >30% chance for obtaining the positive result.

Several laboratories have reported antigen-specific assays, such as the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay, for detecting autoantibodies to GPIIb/IIIa and GPIb, which are either bound to platelet surfaces or present in plasma, although an international study comparing these antigen-specific assays revealed that good inter-laboratory agreement was obtained only when the platelet-associated antibodies were measured [21]. In this regard, Warner and colleagues reported that an antigen-specific assay detecting platelet-associated anti-GPIIb/IIIa antibodies had a sensitivity of 57% and a specificity of 96% for the diagnosis of primary ITP, and the additional measurement of anti-GPIb antibodies increased the diagnostic sensitivity to 66%, and retained a specificity of 92% [3]. In another report using a prospective cohort of thrombocytopenic patients, platelet-associated anti-GPIIb/IIIa and anti-GPIb antibodies detected by direct MAIPA were present in 49% of 93 patients with ITP, including 74 with the primary form, and in only 22% of 54 patients with non-ITP thrombocytopenia [4]. The platelet-associated antibodies to GPIIb/IIIa (88%) were more frequently directed than to GPIb (52%), while 40% of patients had concomitant antibodies to both of these platelet glycoproteins. Finally, McMillan et al examined platelet-associated anti-GPIIb/IIIa and anti-GPIb antibodies in 282 patients with primary ITP, and found that the majority of patient samples contained platelet-associated antibodies recognizing GPIIb/IIIa alone (52%); fewer reacted to GPIb alone (12%) or to both complexes (15%) [5]. Our findings, obtained by measuring the anti-GPIIb/IIIa and anti-GPIb antibody-producing circulating B cells, were generally concordant with these results from assays detecting specific platelet-associated antibodies: GPIIb/IIIa antibodies were predominantly recognized, while anti-GPIb antibody measurement contributed minimally to the diagnosis of primary ITP. The ELISPOT assays appear to be more sensitive than the platelet-associated antigen-specific assays (90% versus 49–66%), but prospective studies comparing the ELISPOT assays with other anti-platelet autoantibody detection tests are necessary to confirm this.

The main reason for the low utility of anti-GPIb antibody-producing B cells for the diagnosis of ITP is that circulating anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells coexist in the majority of patients with ITP. Only a small number of patients had anti-GPIb antibody-producing B cells alone. Therefore, in routine clinical settings, the anti-GPIIb/IIIa ELISPOT assay appears to be sufficient for the diagnosis of ITP. Interestingly, the levels of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in the circulation were correlated with each other in ITP patients, irrespective of whether the diagnosis was primary ITP or one of the secondary forms. These findings indicate that the autoimmune response in the majority of ITP patients targets multiple platelet glycoproteins, which might be a consequence of "epitope spreading" [2]. In this regard, we have proposed a "pathogenic loop" model for the ongoing anti-platelet autoantibody response in ITP patients [22]. Namely, macrophages in the reticuloendothelial system (spleen in the majority of the patients) capture opsonized platelets, and activate autoreactive T helper cells that stimulate the B cells to proliferate [23], differentiate into plasma cells [24], and produce anti-platelet autoantibodies, which in turn bind to circulating platelets. The continuous destruction of platelets in the reticuloendothelial system would allow the processing and presentation of a whole panel of platelet antigens by macrophages, some of which could elicit additional autoreactive T cell responses, resulting in the production of autoantibodies against other platelet glycoproteins.

In primary ITP and various forms of secondary ITP, SLE was unique in having a predominant anti-GPIb antibody-producing B cell response. SLE is a systemic autoimmune disease characterized by a loss of tolerance to nuclear and other self-antigens, a production of pathogenic autoantibodies, and damage to multiple organ systems [25]. Taken together with the association between anti-GPIb antibody-producing B cells and the production of ANAs, even in patients with primary ITP, the anti-GPIb autoantibody response might be linked to systemic autoimmunity.

Because a significant proportion of SLE patients had anti-GPIb antibody-producing B cells in the absence of anti-GPIIb/IIIa antibody-producing B cells, measurement of the anti-GPIb in addition to anti-GPIIb/IIIa antibody-producing B cells may have some merit for accurately identifying secondary ITP in patients with SLE and thrombocytopenia, although the number of patients analyzed in this study was too small to draw a firm conclusion.

Much effort has been made to identify clinical associations of individual anti-platelet glycoprotein antibodies, but the clinical significance of such antibodies remains uncertain. In patients with primary ITP, the presence of platelet-associated anti-GPIb antibodies was shown to be associated with a lower platelet count [26,27] and inadequate responses to corticosteroids [26] and IVIG [28]. Our results were consistent with these previous observations, including the low platelet count and poor responses to therapeutic interventions, especially to IVIG. In this regard, some monoclonal antibodies against GPIb are known to induce platelet activation, which may lead to accelerated platelet destruction independent of the Fcγ receptor-mediated process in ITP patients [29]. We additionally found correlations between anti-GPIb antibody-producing B cells and a low prevalence of *H. pylori* infection or a high frequency of positive ANA. These findings indicate that there may be a relatively homogeneous subset of primary ITP cases defined by the anti-GPIb antibody response, and characterized by severe thrombocytopenia, the absence of *H. pylori* infection, a positive ANA, and a poor therapeutic response.

In summary, our ELISPOT assay for detecting anti-GPIb antibody-secreting B cells is useful for identifying patients with ITP, but its utility for diagnosing ITP is apparently inferior to the anti-GPIIb/IIIa ELISPOT assay. Nevertheless, detection of the anti-GPIb antibody response is useful for subtyping patients with primary ITP and predicting the therapeutic response.

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Author Contributions

Conceived and designed the experiments: MK YI. Performed the experiments: MK YO YI. Analyzed the data: MK YI. Wrote the paper: MK YO YI.

References

1. Stasi R (2001) Immune thrombocytopenia: pathophysiologic and clinical update. Semin Thromb Hemost 38: 454–462.
2. McMillan R (2007) The pathogenesis of chronic immune thrombocytopenic purpura. Semin Hematol 44: S3–S11.
3. Warner MN, Moore JC, Warkentin TE, Santos AV, Kelton JG (1999) A prospective study of protein-specific assays used to investigate idiopathic thrombocytopenic purpura. Br J Haematol 104: 442–447.
4. Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH (1996) Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. Blood 88: 194–201.
5. McMillan R, Wang L, Tani P (2003) Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP). J Thomb Haemost 1: 485–491.
6. Kuwana M, Okazaki Y, Kaburaki J, Kawakami Y, Ikeda Y (2002) Spleen is a primary site for activation of glycoprotein IIb-IIIa-reactive T and B cells in patients with immune thrombocytopenic purpura. J Immunol 168: 3675–3682.
7. Kuwana M, Okazaki Y, Kaburaki J, Ikeda Y (2003) Detection of circulating B cells secreting platelet-specific autoantibody is a sensitive and specific test for the diagnosis of autoimmune thrombocytopenia. Am J Med 114: 322–325.
8. Kuwana M, Kaburaki J, Okazaki Y, Miyazaki H, Ikeda Y (2006) Two types of autoantibody-mediated thrombocytopenia in patients with systemic lupus erythematosus. Rheumatology 45: 851–854.
9. Kajihara M, Kato S, Okazaki Y, kawakami Y, Ishii H, et al (2003) A role of autoantibody-mediated platelet destruction in thrombocytopenia in patients with cirrhosis. Hepatology 37: 1267–1276.
10. Yamazaki R, Kuwana M, Mori T, Okazaki Y, Kawakami Y, et al (2006) Prolonged thrombocytopenia after allogeneic haematopoietic stem cell transplantation: Associations with impaired platelet production and increased platelet turnover. Bone Marrow Transplant 38: 377–384.
11. Kuwana M, Kurata Y, Fujimura K, Fujisawa K, Wada H, et al (2006) Preliminary laboratory-based diagnostic criteria for immune thrombocytopenic purpura: Evaluation by multi-center prospective study. J Thromb Haemost 4: 1936–1943.
12. George JN, Woolf SH, Raskob GE, Wasser JS, Aledort LM, et al (1996) Idiopathic thrombocytopenic purpura: A practice guideline developed by explicit methods for the American Society of Hematology. Blood 88: 3–40.
13. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, et al (2009) Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood 113: 2386–2393.
14. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, et al (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 25: 1271–1277.
15. Erlinger S, Benhamou JP. Cirrhosis: clinical aspects. In: Bircher J, Benhamou JP, McIntyre N, Rizetto M, Rodes J, eds. Oxford textbook of clinical hepatology. Volume 1. 2nd ed. Oxford: Oxford University Press, 1999: 629–41.
16. Guinan EC. (1997) Clinical aspects of aplastic anemia. Hematol Oncol Clin North Am 11: 1025–1044.
17. Greenberg P, Cox C, LeBeau MM, Fanaux P, Morel P, et al (1997) International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 89: 2079–2088.
18. Asahi A, Kuwana M, Suzuki H, Hibi T, Kawakami Y, et al (2006) Effects of a *Helicobacter pylori* eradication regimen on anti-platelet autoantibody response in infected and uninfected patients with idiopathic thrombocytopenic purpura. Haematologica 91: 1436–1437.
19. Kojouri K, Vesely SK, Terrell DR, George JN (2004) Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, prediction of response, and surgical complications. Blood 104: 2623–2634.
20. Murata M, Ware J, Ruggeri ZM (1991) Site-directed mutagenesis of a soluble recombinant fragment of platelet glycoprotein Ibα demonstrating negatively charged residues involved in von Willebrand factor binding. J Biol Chem 266: 15474–15480.
21. Berchtold P, Müller D, Beardsley D, Fujisawa K, Kaplan C, et al (1997) International study to compare antigen-specific methods used for the measurement of antiplatelet autoantibodies. Br J Haematol 96: 477–483.
22. Kuwana M, Okazaki Y, Ikeda Y (2009) Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura. J Thromb Haemost 7: 322–329.
23. Daridon C, Loddenkemper C, Spieckermann S, Kühl AA, Salama A, et al (2011) Splenic proliferative lymphoid nodules distinct from germinal centers are sites of autoantigen stimulation in immune thrombocytopenia. Blood 120: 5021–5031.
24. Mahévas M, Patin P, Huetz F, Descatoire M, Cagnard N, et al (2013) B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells. J Clin Invest 123: 432–442.
25. Liu Z, Davidson A (2012) Taming lupus-a new understanding of pathogenesis is leading to clinical advances. Nat Med 18: 871–882.
26. Nomura S, Yanabu M, Soga T, Kido H, Fukuroi T, et al (1991) Analysis of idiopathic thrombocytopenic purpura patients with antiglycoprotein IIb/IIIa or Ib autoantibodies. Acta Haematol 86: 25–30.
27. Hou M, Stockelberg D, Kutti J, Wadenvik H (1995) Antibodies against platelet GPIb/IX, GPIIb/IIIa, and other platelet antigens in chronic idiopathic thrombocytopenic purpura. Eur J Haematol 55: 307–314.
28. Go RS, Johnston KL, Bruden KC (2007) The association between platelet autoantibody specificity and response to intravenous immunoglobulin G in the treatment of patients with immune thrombocytopenia. Haematologica 92: 283–284.
29. Cauwenberghs N, Schlammadinger A, Vauterin S, Cooper S, Descheemaeker G, et al (2001) Fc-receptor dependent platelet aggregation induced by monoclonal antibodies against platelet glycoprotein Ib or von Willebrand factor. Thromb Haemost 85: 679–685.

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Dysregulated negative immune regulators in immune thrombocytopenia

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Immune thrombocytopenia (ITP) is an autoimmune disease mediated by IgG antiplatelet autoantibodies, resulting in an isolated thrombocytopenia. The mechanism for ongoing antiplatelet antibody production is explained by a 'pathogenic loop' model consisting of macrophages in the reticuloendothelial system, platelet-reactive $CD4^+$ T cells and B cells producing IgG antiplatelet antibodies. In ITP patients, a variety of negative immune regulators including $CD4^+$ T regulatory cells, B regulatory cells and tolerogenic dendritic cells are dysfunctional, resulting in failure to efficiently suppress the pathogenic loop. In addition, *Helicobacter pylori* infection leads to defective inhibitory FcγRIIB signalling in macrophages and thereby increases susceptibility to ITP. In ITP patients, dysregulation of these negative immune regulators is associated with each other in the impaired immune regulatory network. Thus, strategies that enhance functions of these intrinsic negative immune regulators would be promising future approaches for treating ITP.

Key words: autoantibody, immune thrombocytopenia, regulatory T cells

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disease that causes an isolated thrombocytopenia. ITP can occur either alone or in the setting of other immune-mediated disorders such as systemic lupus erythematosus and human immunodeficiency virus infection. Mechanisms for thrombocytopenia in ITP patients include increased platelet consumption and impaired platelet production, both of which are mediated mainly through IgG antiplatelet autoantibodies [1]. The major autoimmune targets are platelet membrane glycoproteins, such as GPIIb/IIIa and GPIb/IX [2]. It has been believed that ITP is associated with a loss of tolerance to platelet antigens, but triggers that break immune tolerance still remain unclear. There are accumulating lines of evidence demonstrating that cellular immunity in ITP patients is perturbed and shifted towards T helper 1 (Th1) and T helper 17 (Th17) proinflammatory responses [3]. However, autoimmune responses observed in patients with ITP, especially those with primary ITP, specifically target platelet antigens. Therefore, antigen-specific mechanisms should play a critical role in the pathogenesis in addition to the activation of non-specific immune pathways. It is widely appreciated that the production of IgG autoantibodies requires isotype switch and affinity maturation mediated by autoantigen-specific $CD4^+$ T cells. In fact, in patients with ITP, we have identified $CD4^+$ T cells reactive to GPIIb/IIIa, which are able to stimulate B cells to produce IgG antibodies that are able to bind normal platelet surfaces at least *in vitro* [4]. Therefore, collaboration between antigen-specific and non-specific immune mechanisms is necessary to fully emerge antiplatelet autoantibody responses.

It has been known that autoreactive T cells escape from apoptosis during the negative selection process in the thymus and are a component of the normal T-cell repertoire. These potentially harmful autoreactive T cells are suppressed or deleted in a variety of negative immune regulators in periphery. These negative regulators play a pivotal role in the homeostasis of the immune system and have emerged as key players in the development and maintenance of peripheral immune tolerance. Several immune cell subsets have been reported to possess such immunosuppressive function, and include T regulatory

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cells (Tregs), B regulatory cells (Bregs) and tolerogenic dendritic cells (DCs). In addition, a variety of soluble mediators, receptors and intracellular signalling molecules are known as negative immune regulators. These include interleukin (IL)-10, transforming growth factor-β (TGF-β), Fcγ receptor (FcγR) IIB, cytotoxic T-lymphocyte antigen 4 (CTLA4), indoleamine-2,3-dioxygenase-1 (IDO1) and suppressor of cytokine signalling (SOCS) family proteins. It has been shown that deficiency of these negative regulators leads to emergence of autoimmunity in mice, and single nucleotide polymorphisms with the genes for many negative regulators are associated with susceptibility to autoimmune diseases. Recent accumulating lines of evidence have shown involvement of dysregulated negative immune regulators in pathogenic processes of ITP. In addition, a series of studies on ITP patients have reported the improvement of quantity and/or function of negative immune regulators after platelet recovery with treatment, suggesting that they might be preferable therapeutic targets. This review summarizes updated knowledge on roles of negative immune regulators in the pathogenic process of ongoing antiplatelet autoantibody production in ITP patients.

A 'pathogenic loop model' for ITP pathogenesis

Ongoing antiplatelet autoantibody response observed in ITP patients requires sustained activation of platelet-reactive $CD4^+$ T cells by recognizing antigenic peptides efficiently presented by antigen-presenting cells. Interestingly, GPIIb/IIIa-reactive $CD4^+$ T cells responded to tryptic peptides of GPIIb/IIIa or recombinant GPIIb/IIIa fragments produced in bacteria, but not to native GPIIb/IIIa [5, 6], indicating that the epitopes they recognize are 'cryptic' determinants, which are generated at a subthreshold level by the processing of native GPIIb/IIIa under normal circumstances. Therefore, exposure of 'cryptic' peptides of GPIIb/IIIa to the immune system is a critical step for maintaining the antiplatelet autoantibody response. By using GPIIb/IIIa-reactive $CD4^+$ T-cell lines and freshly isolated splenocytes from the same ITP patients, we were able to identify splenic macrophages as the major antigen-presenting cells for presenting 'cryptic' GPIIb/IIIa peptides *in vivo* [7]. In addition, presentation of the 'cryptic' GPIIb/IIIa peptides by macrophages depended on their phagocytosis of opsonized platelets via FcγRs. Since the spleen is the primary site of activation of GPIIb/IIIa-reactive $CD4^+$ T cells and subsequent antiplatelet antibody production [8], it is likely that splenic macrophages that take up a large number of opsonized platelets via FcγRs efficiently concentrate the small quantities of platelet antigens that were previously 'cryptic'. A recent study evaluating detailed histologic analysis of ITP spleens indicates that splenic proliferative lymphoid nodules are the primary sites of the autoantigenic stimulation in ITP patients [9]. Based on the results from our *in vitro* assay for anti-GPIIb/IIIa antibody production, we have proposed a 'pathogenic loop' model for the ongoing IgG antiplatelet autoantibody response in ITP patients [7]. Namely, macrophages in the reticuloendothelial system capture opsonized platelets via cell surface FcγRs and present antigenic platelet-derived peptides to T cells in the context of the major histocompatibility complex (MHC) molecule. Autoreactive $CD4^+$ T cells to GPIIb/IIIa are then activated by recognition of the antigenic peptides and exert helper activity to stimulate B cells to produce IgG antiplatelet autoantibodies, which in turn bind to circulating platelets. Theoretically, once this pathogenic loop is established, production of IgG antiplatelet antibodies goes on endlessly, irrespective of triggers that initiated this response. The majority of current treatment regimens for ITP are aimed to interrupt this pathogenic loop, thereby suppressing the production of IgG antiplatelet antibodies and resultant platelet recovery. Specifically, corticosteroids suppress overall immune responses, while splenectomy removes the major site for this loop. Cytotoxic immunosuppressants, such as cyclophosphamide and azathioprine, inhibit the proliferation of both T and B cells, while cyclosporin selectively inhibits T-cell activation and rituximab depletes the entire B cells in circulation. Therefore, any interventions capable of inhibiting this pathogenic loop are potentially effective for ITP.

$CD4^+$ Tregs

$CD4^+$ Tregs are heterogeneous in terms of cell surface phenotypes, cytokine production profiles and mechanisms for action [10]. The $CD4^+$ T cells with immunosuppressive capacity, which are produced in the thymus and are delivered to the periphery, are called naturally occurring Tregs. This Treg subset expresses a master transcription factor FoxP3 as well as a high level of CD25. On the other hand, $CD4^+$ T cells that acquire regulatory properties under particular conditions in periphery are called adaptive Tregs. Of heterogeneous adaptive Treg subsets, $FoxP3^+$ Tregs with a phenotype nearly identical to naturally occurring Tregs are called induced Tregs. Mechanisms of immune suppression by $FoxP3^+$ Tregs, including both naturally occurring and induced Tregs, are not fully elucidated, but secretion of immunosuppressive cytokines, such as TGF-β, IL-10 and IL-35, and interruption of the CD80/CD86-CD28 signal with CTLA4 are thought to be main mechanisms [10]. Blockade of the critical costimulatory signal between CD80/CD86 and CD28 during a cognate interaction between T cells and antigen-presenting

cells results in anergy of T cells. In addition, CTLA4 expressed on Tregs modulates functions of specialized antigen-presenting cells such as DCs and induces tolerogenic DCs through maturation inhibition, down-regulation of CD80/CD86 and induction of an immune regulatory enzyme IDO1.

Given a critical role of FoxP3$^+$ Tregs in preventing the autoimmune response [11], dysregulation of Tregs could also be associated with pathophysiology of ITP. In fact, a number of studies demonstrated a decreased proportion of FoxP3$^+$ Tregs in peripheral blood CD4$^+$ T cells in ITP patients than in healthy controls [12]. Treg frequency was also decreased in bone marrow and spleen of ITP patients [9]. Some studies failed to detect differences in Treg proportions between ITP patients and healthy controls, but this is explained partly by the use of different phenotypes for the identification of Tregs. On the other hand, Treg's ability to suppress allogeneic T-cell response was shown to be inferior in ITP patients than in healthy controls. Several studies evaluated serial changes in Treg proportion and function before and after treatment. High-dose dexamethasone and rituximab increased the proportion of Tregs in responders [13, 14], whereas thrombopoietin receptor agonists failed to increase Treg proportion, but improved Treg function [15].

We have demonstrated that FoxP3$^+$ Treg-deficient mice spontaneously develop chronic thrombocytopenia with apparent bleeding tendency [16]. In this model, transfer of FoxP3$^+$ Tregs completely prevented the onset of thrombocytopenia, indicating that deficiency in FoxP3$^+$ Tregs is responsible for emergence of thrombocytopenia. Moreover, Treg's protective effect against thrombocytopenia was completely cancelled by the administration of anti-CTLA4 neutralizing antibody. IgG antibodies capable of binding to platelet surfaces were detected specifically in platelet eluates from thrombocytopenic mice. Later, the primary target of antiplatelet autoantibodies produced in FoxP3$^+$ Treg-deficient mice was identified as GPIb/IX [17], indicating that thrombocytopenia observed in FoxP3$^+$ Treg-deficient mice is mediated through the production of IgG antiplatelet autoantibodies, which is analogous to human ITP. A series of experiments using another ITP mouse model have demonstrated that peripheral Treg deficiency is caused mainly by thymic retention [18]. Taken together, these animal studies support a critical role of FoxP3$^+$ Tregs in preventing emergence of autoimmunity that results in the production of IgG autoantibodies reactive with platelet antigens.

Given a critical role of FoxP3$^+$ Tregs in suppressing autoreactive CD4$^+$ T cells directly through the expression of immunosuppressive cytokines and CTLA4 as well as indirectly through the induction of tolerogenic DCs, excess quantity or function of FoxP3$^+$ Tregs would interrupt the pathogenic loop for ITP. In this regard, two recent studies have shown that administration of low-dose IL-2 leads to mobilization and activation of FoxP3$^+$ Tregs and subsequent clinical improvement in patients with chronic graft-versus-host disease or in those with vasculitis related to hepatitis C virus infection [19, 20], although adoptive transfer of FoxP3$^+$ Tregs failed to increase platelet count in FoxP3$^+$ Treg-deficient mice after onset of thrombocytopenia [16].

Bregs (B10 cells)

Recently, B cells with capacity to negatively regulate cellular immune responses have been described, and the concept of Bregs has emerged [21]. The deficiency of Breg is associated with worsening of a variety of autoimmune diseases, such as systemic lupus erythematosus and experimental autoimmune encephalomyelitis, in mouse models [22]. Bregs are a heterogeneous cell population like Tregs and inhibit cellular immune responses and inflammation, especially Th1 responses. Interestingly, it has been shown that Bregs are able to promote differentiation and recruitment of Tregs. Mechanisms of immune suppression by Bregs are not fully clarified, but IL-10-producing subset known as B10 cells has been extensively analysed [22]. B10 cells are a functionally defined subset currently identified only by their competency to produce a large amount of IL-10 following appropriate stimulation such as toll-like receptors and CD40 ligand. Although B10 cells share surface markers with other previously defined B-cell subsets, B10 cells are enriched in the CD19$^+$CD24^{high}CD38^{high} B-cell subset [23]. Li *et al.*[24] recently found a lower frequency of circulating B10 cells in non-splenectomized patients with ITP. These B10 cells were functionally impaired in their ability to produce IL-10 in response to appropriate stimulation. B10 cells were increased after platelet recovery by treatment with thrombopoietin receptor agonists. These data indicate a dysregulated Breg subset as an additional defect in the immune network in ITP patients. Expanding the immunosuppressive properties of B10 cells can provide a new approach to the treatment of ITP by interrupting the pathogenic loop by suppressing differentiation and activation of platelet-specific CD4$^+$ T cells. Theoretically, manipulation of this subset is possible by selective expansion of B10 cells, although it is currently infeasible to selectively target B10 cells because of the lack of surface molecule or ligand for expansion specific for this subset.

Tolerogenic DCs

Despite strong immunogenic ability of DCs in eliciting immune responses, they have also been ascribed opposite

roles in maintenance of tolerance and suppression of adaptive immune responses [25]. Mechanisms how DCs maintain a fine balance between tolerance and immunity are explained by different functional capacities in their maturation status. DCs are required to deliver two signals simultaneously to fully stimulate T cells: the MHC–peptide complex and a series of costimulatory signals including CD80/CD86-CD28 and CD40-CD154. Production of proinflammatory cytokines such as IL-12 during the DC–T-cell interaction is also critical. Immature or semi-mature DCs lacking sufficient expression of costimulatory molecules and inflammatory cytokines are incapable of stimulating T cells, but induce anergy of T cells and induction of adaptive Tregs, and thus are called tolerogenic DCs [26]. Animal studies have demonstrated that adoptive transfer of tolerogenic DCs can induce antigen-specific Tregs and prevent autoimmune diseases [27]. Plasmacytoid DC (pDC) is one of the circulating DC subsets that produces a large quantity of type 1 interferon in response to CpG-containing DNA viruses and bacteria. The majority of circulating pDCs are known to be immature in normal circumstances and function as tolerogenic DCs [28]. Recently, Saito *et al.* [29] reported that circulating pDCs were decreased in patients with primary ITP and *H. pylori*-associated ITP, compared to age- and sex-matched healthy controls. In addition, patients who achieved platelet recovery after *H. pylori* eradication experienced increase in circulating pDCs, whereas patients who did not respond to the treatment continued to have a low pDC count. Furthermore, it has been shown that function of tolerogenic DCs is also impaired in ITP patients. Specifically, IDO1 expression was decreased in circulating DCs from ITP patients, resulting in a reduced capacity to induce adaptive Tregs [30].

Tolerogenic DCs have potential utility for treating autoimmune diseases and transplant rejection [31]. One of the merits to use tolerogenic DCs is their ability to induce antigen-specific tolerance. Specifically, antigen-captured tolerogenic DCs are likely to be capable of suppressing the pathogenic loop of ITP by anergizing platelet-specific $CD4^+$ T cells. In addition, adaptive Tregs specific to platelet antigens induced by the interaction with tolerogenic DCs may further suppress antiplatelet autoantibody response. Many protocols for *ex vivo* generation of tolerogenic DCs have been reported to date. These manufactured tolerogenic DCs expressed high levels of inhibitory receptors such as ILT-3 and ILT-4 and have capacity to induce anergy in potentially harmful auto- or alloreactive T cells and/or to generate antigen-specific adaptive Tregs [32]. The tolerogenic DC induction cultures required the addition of one or a combination of IL-10, TGF-β, vitamin D3, dexamethasone, rapamycin and mycophenolate mofetil. Molecular biology techniques such as RNA interference technology are also utilized to down-regulate some proinflammatory signals, for example IL-12 and NF-κB components [33]. On the other hand, overexpression of inhibitory molecules (e.g. IL-10, TGF-β, CTLA4 and SOCS1) induces tolerogenic DCs. Another potential strategy to induce antigen-specific Tregs is blockade of critical costimulatory signals during the antigen-dependent interaction between T cells and antigen-presenting cells [34]. We have previously shown that repeated treatment with anti-CD154 monoclonal antibody during antigenic stimulation of GPIIb/IIIa-specific $CD4^+$ T-cell lines results in the generation of GPIIb/IIIa-specific Tregs, which are anergic and express a high level of IL-10 [35]. These antigen-specific Tregs actually suppressed the activation of effector GPIIb/IIIa-specific $CD4^+$ T cells and subsequent antiplatelet antibody production in a co-culture with autologous B cells in a dose-dependent manner.

FcγRIIB signalling

FcγRs are cell surface receptor for IgG and are expressed by haematopoietic cells with capacity of clearing opsonized antigens, such as monocytes/macrophages, neutrophils and B cells [36]. There are three activating receptors, including FcγRI, FcγRIIA and FcγRIII, in humans, while FcγRIIB is a sole inhibitory receptor. These FcγRs control immune responses through a balance between activating and inhibitory receptors. In mice, FcγRIIB deficiency is associated with increased inflammation, allergy and development of chronic autoimmunity [37]. It has been reported that FcγRIIB expression on B cells and DCs is important for the induction of antigen-specific immune tolerance through the induction of adaptive Tregs [38]. There is growing evidence that the eradication of *Helicobacter pylori* (*H. pylori*) effectively increases platelet count in a considerable proportion of ITP patients infected with this bacterium [39]. The strong relationship between platelet recovery and disappearance of *H. pylori* indicates a direct role of *H. pylori* infection in pathogenic process of ITP. We have recently found that *H. pylori* infection modulates the FcγR balance of monocytes/macrophages in favour of activating FcγRs, through down-regulation of the inhibitory FcγRIIB [40]. Specifically, circulating monocytes from *H. pylori*-infected patients exhibit enhanced phagocytic capacity and low expression levels of inhibitory FcγRIIB. This activated monocyte phenotype was suppressed 1 week after starting the *H. pylori* eradication regimen. The antiplatelet autoantibody responses and platelet kinetic parameters subsequently improved, indicating that suppression of the activated monocyte function precedes the improvement in the autoantibody response. Interestingly, a change in FcγR balance towards the inhibitory FcγRIIB in monocytes/macrophages has

also reported in the therapeutic action of other established treatment regimens for ITP, such as intravenous immunoglobulin [41] and high-dose dexamethasone [42]. These findings together indicate that the FcγR balance of monocytes/macrophages is an attractive therapeutic target for ITP. In this case, fostamatinib, a Syk inhibitor that blocks the downstream signal of activating FcγRs, was shown to be efficacious in increasing platelet count in ITP patients in a phase 2 clinical trial [43].

Conclusions and further prospects

In ITP patients, a variety of intrinsic negative immune regulators are dysfunctional (Fig. 1). Reduced number and/or impaired function of $CD4^+$ Tregs, Bregs (B10 cells) and tolerogenic DCs may contribute to enhance the pathogenic loop of ITP. In addition, *H. pylori* infection leads to defective inhibitory FcγRIIB signalling in macrophages and thereby increases susceptibility to ITP. Interestingly, dysregulation of these negative immune regulators is associated with each other in the immune regulatory networks. For example, $CD4^+$ Tregs are capable of inducing tolerogenic DCs via the cell surface CTLA4. On the other hand, tolerogenic DCs have ability to induce adaptive Tregs, and this process is promoted by Bregs. In addition, FcγRIIB expressed by B cells and DCs is involved in the induction of adaptive Tregs. Further studies evaluating the mechanisms for dysregulation of intrinsic negative immune regulators in ITP patients are necessary to elucidate the pathogenesis of ITP. Furthermore, treatment with immunosuppressive agents remains the mainstay of ITP therapies, but modulation of intrinsic negative immune regulators should be another therapeutic option with a low risk of excessive immunosuppression.

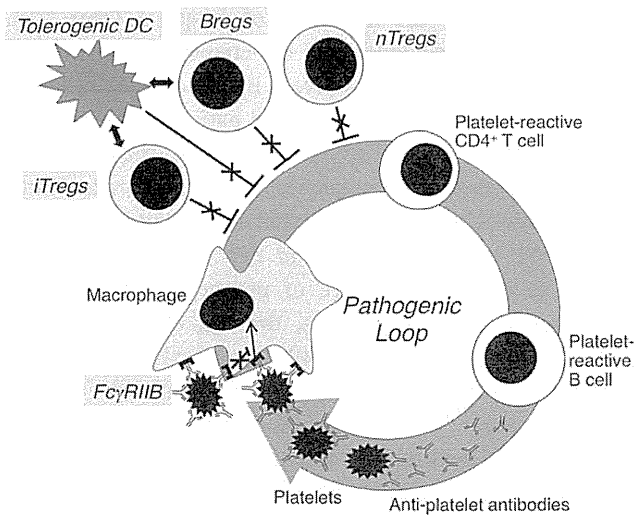

Fig. 1 Defective negative immune regulators and a continuous pathogenic loop carried out by macrophages in the reticuloendothelial system, autoreactive $CD4^+$ T cells and antibody-producing B cells that maintains antiplatelet autoantibody production in ITP patients. Breg, B regulatory cells; DC, dendritic cell; iTreg, induced T regulatory cells; nTreg, naturally occurring T regulatory cells.

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Masataka Kuwana drafted the paper and approved it for submission and final version.

Disclosure

The authors have no conflict of interest to declare.

References

1 Johnsen J: Pathogenesis in immune thrombocytopenia: new insights. *Hematology Am Soc Hematol Educ Program* 2012; 2012:306–312
2 McMillan R: Autoantibodies and autoantigens in chronic immune thrombocytopenic purpura. *Semin Hematol* 2000; 37:239–248
3 Semple JW, Provan D: The immunopathogenesis of immune thrombocytopenia: T cells still take center-stage. *Curr Opin Hematol* 2012; 19:357–362
4 Kuwana M, Kaburaki J, Ikeda Y: Autoreactive T cells to platelet GPIIb-IIIa in immune thrombocytopenic purpura: role in production of anti-platelet autoantibody. *J Clin Invest* 1998; 102:1393–1402
5 Kuwana M, Kaburaki J, Kitasato H, *et al.*: Immunodominant epitopes on glycoprotein IIb-IIIa recognized by autoreactive T cells in patients with immune thrombocytopenic purpura. *Blood* 2001; 98:130–139
6 Kuwana M, Ikeda Y: The role of autoreactive T-cells in the pathogenesis of ITP. *Int J Hematol* 2005; 81:106–112
7 Kuwana M, Okazaki Y, Ikeda Y: Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura. *J Thromb Haemost* 2009; 7:322–329
8 Kuwana M, Okazaki Y, Kaburaki J, *et al.*: Spleen is a primary site for activation of platelet-reactive T and B cells in patients with immune thrombocytopenic purpura. *J Immunol* 2002; 168:3675–3682
9 Daridon C, Loddenkemper C, Spieckermann S, *et al.*: Splenic proliferative lymphoid nodules distinct from germinal centers are sites of autoantigen stimulation in immune thrombocytopenia. *Blood* 2012; 120:5021–5031
10 Sakaguchi S, Miyara M, Costantino CM, *et al.*: FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 2010; 10:490–500

11 Miyara M, Gorochov G, Ehrenstein M, *et al.*: Human FoxP3 + regulatory T cells in systemic autoimmune diseases. *Autoimmun Rev* 2011; **10**:744–755

12 Nishimoto T, Kuwana M: CD4 + CD25 + Foxp3 + regulatory T cells in pathophysiology of immune thrombocytopenia. *Semin Hematol* 2013; **50**:S43–S49

13 Ling Y, Cao X, Yu Z, *et al.*: Circulating dendritic cells subsets and CD4 + Foxp3 + regulatory T cells in adult patients with chronic ITP before and after treatment with high-dose dexamethasone. *Eur J Haematol* 2007; **79**:310–316

14 Stasi R, Cooper N, Del Poeta G, *et al.*: Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. *Blood* 2008; **112**:1147–1150

15 Bao W, Bussel JB, Heck S, *et al.*: Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents. *Blood* 2010; **116**:4639–4645

16 Nishimoto T, Satoh T, Takeuchi T, *et al.*: Critical role of CD4 (+)CD25(+) regulatory T cells in preventing murine autoantibody-mediated thrombocytopenia. *Exp Hematol* 2012; **40**:279–289

17 Nishimoto T, Satoh T, Simpson EK, *et al.*: Predominant autoantibody response to GPIb/IX in a regulatory T-cell-deficient mouse model for immune thrombocytopenia. *J Thromb Haemost* 2013; **11**:369–372

18 Aslam R, Hu Y, Gebremeskel S, *et al.*: Thymic retention of CD4 + CD25 + FoxP3 + T regulatory cells is associated with their peripheral deficiency and thrombocytopenia in a murine model of immune thrombocytopenia. *Blood* 2012; **120**:2127–2132

19 Koreth J, Matsuoka K, Kim HT, *et al.*: Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* 2011; **365**:2055–2066

20 Saadoun D, Rosenzwajg M, Joly F, *et al.*: Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 2011; **365**:2067–2077

21 Bouaziz JD, Yanaba K, Tedder TF: Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol Rev* 2008; **224**:201–214

22 Kalampokis I, Yoshizaki A, Tedder TF: IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. *Arthritis Res Ther* 2013; **15**(Suppl 1):S1

23 Blair PA, Norena LY, Flores-Borja F, *et al.*: CD19(+)CD24(hi) CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. *Immunity* 2010; **32**:129–140

24 Li X, Zhong H, Bao W, *et al.*: Defective regulatory B cell compartment in patients with immune thrombocytopenia. *Blood* 2012; **16**:3318–3325

25 Merad M, Sathe P, Helft J, *et al.*: The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 2013; **31**:563–604

26 Saei A, Hadjati J: Tolerogenic dendritic cells: key regulators of peripheral tolerance in health and disease. *Int Arch Allergy Immunol* 2013; **161**:293–303

27 Ganguly D, Haak S, Sisirak V, *et al.*: The role of dendritic cells in autoimmunity. *Nat Rev Immunol* 2013; **13**:566–577

28 Bronte V: Tolerogenic pDCs: spotlight on Foxo3. *J Clin Invest* 2011; **121**:1247–1250

29 Saito A, Yokohama A, Osaki Y, *et al.*: Circulating plasmacytoid dendritic cells in patients with primary and *Helicobacter pylori*-associated immune thrombocytopenia. *Eur J Haematol* 2012; **88**:340–349

30 Catani L, Sollazzo D, Trabanelli S, *et al.*: Decreased expression of indoleamine 2,3-dioxygenase 1 in dendritic cells contributes to impaired regulatory T cell development in immune thrombocytopenia. *Ann Hematol* 2013; **92**:67–78

31 Van Brussel I, Lee WP, Rombouts M, *et al.*: Tolerogenic dendritic cell vaccines to treat autoimmune diseases: can the unattainable dream turn into reality? *Autoimmun Rev* 2014; **13**:138–150

32 Manavalan JS, Rossi PC, Vlad G, *et al.*: High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. *Transpl Immunol* 2003; **11**:245–258a

33 Li R, Zheng X, Popov I, *et al.*: Gene silencing of IL-12 in dendritic cells inhibits autoimmune arthritis. *J Transl Med* 2012; **10**:19

34 Kuwana M, Kaburaki J, Wright TM, *et al.*: Induction of antigen-specific human $CD4^+$ T cell anergy by peripheral blood DC2 precursors. *Eur J Immunol* 2001; **31**:2547–2557

35 Kuwana M, Kawakami Y, Ikeda Y: Suppression of autoreactive T-cell response to glycoprotein IIb/IIIa by blockade of CD40/CD154 interaction: implications for treatment of immune thrombocytopenic purpura. *Blood* 2003; **101**:621–623

36 Takai T: Roles of Fc receptors in autoimmunity. *Nat Rev Immunol* 2002; **2**:580–592

37 Smith KG, Clatworthy MR: FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. *Nat Rev Immunol* 2010; **10**:328–343

38 Sun JB, Xiang Z, Smith KG, *et al.*: Important role for FcγRIIB on B lymphocytes for mucosal antigen-induced tolerance and Foxp3 + regulatory T cells. *J Immunol* 2013; **191**:4412–4422

39 Kuwana M: *Helicobacter pylori*-associated immune thrombocytopenia: clinical features and pathogenic mechanisms. *World J Gastroenterol* 2014; **20**:714–723.

40 Asahi A, Nishimoto T, Okazaki Y, *et al.*: *Helicobacter pylori* eradication shifts monocytes' Fcγ receptor balance toward inhibitory FcγRIIB in immune thrombocytopenic purpura. *J Clin Invest* 2008; **118**:2939–2949

41 Samuelsson A, Towers TL, Ravetch JV: Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science* 2001; **291**:484–486

42 Liu XG, Ma SH, Sun JZ, *et al.*: High-dose dexamethasone shifts the balance of stimulatory and inhibitory Fcgamma receptors on monocytes in patients with primary immune thrombocytopenia. *Blood* 2011; **117**:2061–2069

43 Podolanczuk A, Lazarus AH, Crow AR, *et al.*: Of mice and men: an open-label pilot study for treatment of immune thrombocytopenic purpura by an inhibitor of Syk. *Blood* 2009; **113**:3154–3160

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RAPID COMMUNICATION

Induction of immune tolerance to platelet antigen by short-term thrombopoietin treatment in a mouse model of immune thrombocytopenia

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Abstract Immune thrombocytopenia (ITP) is an autoimmune disorder caused by IgG anti-platelet autoantibodies. Thrombopoietin (TPO) receptor agonists are highly effective in inducing the recovery of platelet counts in ITP patients. Although these agents are thought to promote platelet production without affecting the autoimmune pathogenesis of the disease, a small subset of ITP patients exhibits sustained platelet recovery after treatment termination. To investigate mechanisms involved in this sustained recovery, we evaluated the effects of short-term TPO treatment using a mouse ITP model generated by $Foxp3^+$ T regulatory cell (Treg) depletion. After treatment, platelet recovery was sustained, along with complete suppression of both anti-platelet autoantibody production and T-cell responses to platelet autoantigens. TPO treatment also promoted the peripheral induction of $Foxp3^+$ Tregs in conjunction with elevated circulating TGF-β levels. In summary, thrombopoietic agents are capable of inducing immune tolerance to platelet autoantigens, thereby suppressing the autoimmune pathogenesis of ITP.

Keywords Immune thrombocytopenia · Immune tolerance · Platelets · T regulatory cells · Thrombopoietin

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Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder caused by the production of IgG autoantibodies to platelet membrane glycoproteins, such as GPIIb/IIIa and GPIb [1], which depends on activation of pathogenic autoreactive $CD4^+$ T cells [2]. In ITP patients, various immunosuppressive processes, including T regulatory cell (Treg)-mediated immune regulation, are dysfunctional, resulting in autoreactive T-cell activation [3]. We recently demonstrated that $Foxp3^+$ Treg-deficient mice spontaneously develop sustained thrombocytopenia associated with IgG anti-GPIb antibody production [4, 5], analogous to the pathophysiology of human ITP. This mouse model is useful for evaluating novel therapeutic strategies for ITP.

Thrombopoietin (TPO) regulates thrombopoiesis through the activation of megakaryocytes in the bone marrow, resulting in increased platelet production [6]. Recently, the development of TPO receptor agonists (TPO-RAs), such as romiplostim and eltrombopag, was a significant breakthrough in ITP treatment [6]. It is thought that TPO-RAs do not affect the autoimmune pathogenesis of ITP, since platelet counts typically drop to pre-treatment levels immediately after treatment termination. However, recent reports showed that platelet recovery was unexpectedly sustained in some patients even after TPO-RA was discontinued [7–9]. The exact prevalence of TPO-RA-induced sustained remission is unclear, but one report described that this occurred in 3 of 31 patients treated with TPO-RAs [9]. Based on this observation, a single course of dexamethasone in combination with eltrombopag has been evaluated for a potential therapeutic strategy that induces long-term remission in newly diagnosed ITP patients [10]. Here, we investigated mechanisms mediating the sustained

effects of thrombopoietic agents using our mouse ITP model.

Materials and methods

Recombinant TPO (rTPO) treatment of Treg-deficient ITP mice

Treg-deficient mice were established by transferring $CD4^{+-}$ $CD25^{-}$ T cells into syngeneic nude mice, as previously described [4]. Four weeks after transfer, ITP mice, which were confirmed to have thrombocytopenia (platelet count $\leq 0.33 \times 10^6/\mu L$), were treated with intravenous administration of mouse rTPO (300 ng/mouse; kindly provided by Kyowa Hakko Kirin, Tokyo, Japan) or vehicle for five consecutive days. Experimental protocols were approved by the Keio University Ethics Committee for Animal Experiments.

Evaluation of autoantibody production and autoreactive T-cell responses in splenocytes

The production of pathogenic IgG anti-platelet antibodies in splenocyte cultures was assessed using a platelet/IgG-binding assay, as described previously [4]. The T-cell response to GPIbα was measured as previously described [2], with some modifications. Briefly, five different overlapping regions encompassing all 734 amino acid residues (aar) of GPIbα were expressed and purified as recombinant maltose-binding protein (MBP) fusion proteins [11]. These included α1 (aar 18–250), α2 (aar 242–400), α3 (aar 399–570), α4 (aar 558–674), and α5 (aar 666–734). The splenocytes were cultured in triplicate with the recombinant GPIbα fragments or MBP (5 μg/mL) for 7 days. T-cell proliferation was determined by ^{3}H-thymidine incorporation, and the antigen-specific T-cell response was expressed as a stimulation index (SI), calculated as the ratio of the cpm incorporated in the presence of each GPIbα fragment to the cpm incorporated with MBP. Phytohemagglutinin (PHA) was used to demonstrate non-specific T-cell responsiveness.

Detection of $Foxp3^+$ Tregs

Splenocytes were fixed, permeabilized, and incubated with fluorescence-conjugated monoclonal antibodies to CD4 (BD Biosciences, San Diego, CA, USA), CD25 (BD Biosciences), and Foxp3 (eBioscience, San Diego, CA, USA) [4]. The proportion of $CD25^{high}Foxp3^+$ cells gated in the $CD4^+$ cell fraction was recorded as $Foxp3^+$ Tregs. The cells were analyzed on a FACSCalibur® flow cytometer (BD Biosciences) using CellQuest software.

Transforming growth factor (TGF)-β measurement

The TGF-β concentration in platelet-poor plasma was measured using the Luminex assay (Life Technology, Grand Island, NY, USA) according to the manufacturer's instructions.

Statistical analyses

Continuous variables are shown as the mean ± standard deviation. Comparisons between two groups were tested for statistical significance using the nonparametric Mann–Whitney *U* test.

Results and discussion

Treg-deficient ITP mice were treated with rTPO or vehicle and followed until week 10 (Fig. 1a). Platelet counts gradually increased after rTPO administration and peaked at week 7. After this time point, platelet counts started to decrease, but increased again at week 9 and remained high at week 10. Long-term observation of two rTPO-treated mice revealed that platelet recovery persisted for more than 20 weeks. Previous studies showed that increased platelet counts in rTPO-treated normal mice were no longer detectable 2 weeks after treatment [12] and that spontaneous platelet recovery was not observed in the ITP mouse model [4]. Thus, our finding, combined with those of previous reports, suggested that rTPO may exert platelet-increasing effects other than the direct stimulation of platelet production. To examine rTPO's effects on immune regulation, we prepared splenocytes from rTPO- or mock-treated ITP mice at week 10 and evaluated their autoantibody production (Fig. 1b). While splenocytes from mock-treated mice spontaneously produced pathogenic IgG anti-platelet antibodies, those from rTPO-treated mice did not. This is consistent with a previous report showing that short-term treatment of male (NZW × BXSB) F1 mice, another ITP model, with rTPO resulted in reduction of platelet-associated IgG [13]. Next, we evaluated the autoreactive T-cell responses to a series of recombinant GPIbα fragments (Fig. 1c). Splenic T cells from mock-treated mice proliferated in response to GPIbα fragments, whereas those from rTPO-treated ITP mice showed no detectable response, but did respond to the mitogenic stimulation with PHA. Together, these findings indicate that short-term treatment of ITP mice with thrombopoietic agents not only stimulates platelet production, but also induces immune tolerance to platelet autoantigens.

In our mouse model, transferred conventional $CD4^+$ T cells, which were confirmed to lack $Foxp3^+$ Tregs, expand rapidly through homeostatic proliferation, while

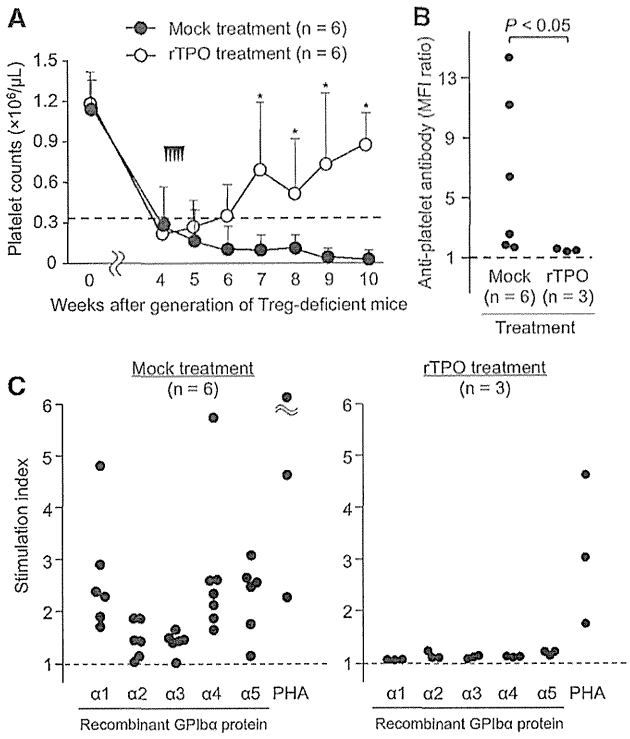

Fig. 1 Short-term treatment with rTPO in a Treg-deficient mouse ITP model. **a** Serial platelet counts in rTPO- or vehicle (mock)-treated ITP mice. The broken line indicates the cutoff level for thrombocytopenia, defined as a platelet count of $0.33 \times 10^6/\mu L$, and the arrowheads denote the timing of administration of rTPO or vehicle. *Asterisks* denote statistically significant differences between rTPO- and mock-treated mice. **b** IgG anti-platelet antibodies in the supernatants of splenocyte cultures derived from rTPO- and mock-treated mice at week 10. Antibody levels were determined by flow cytometry and are shown as the mean fluorescent index (MFI) ratio. **c** T-cell proliferative response to recombinant GPIbα fragments in splenocyte cultures derived from rTPO- and mock-treated mice at week 10. Phytohemagglutinin (PHA) was used to demonstrate non-specific T-cell responsiveness

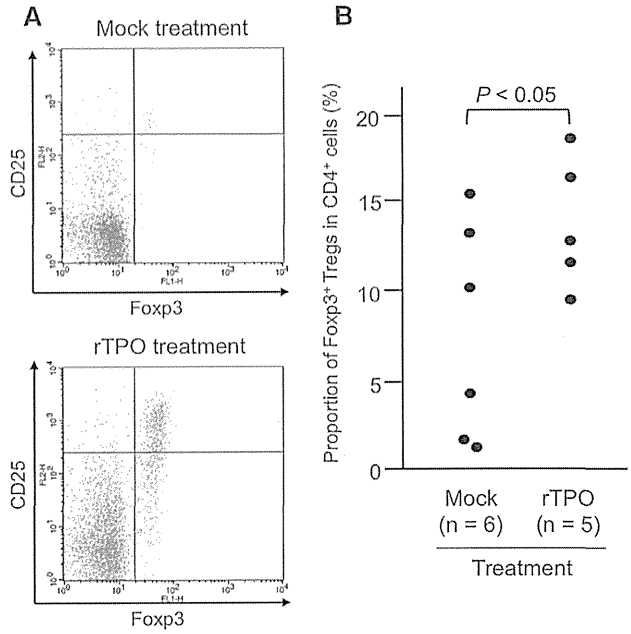

Fig. 2 Proportion of $Foxp3^+$ Tregs in splenocytes derived from rTPO- and mock-treated ITP mice at week 10. **a** A representative dot plot analysis of $CD25^{high}Foxp3^+$ cells gated in the $CD4^+$ cell fraction. The upper-right quadrant corresponds to $Foxp3^+$ Tregs. **b** Proportion of $Foxp3^+$ Tregs in rTPO- and mock-treated ITP mice

the transfer of Treg-deficient $CD4^+$ cells results in the expansion of autoreactive T cells leading to a harmful anti-platelet autoimmune response [4]. In addition, adaptive $Foxp3^+$ Tregs can be peripherally induced from naive $CD4^+$ T cells in the presence of IL-2 and TGF-β [3]. To examine rTPO's effect on this process in ITP mice, $Foxp3^+$ Tregs were evaluated using splenocytes derived from rTPO- and mock-treated ITP mice at week 10. $Foxp3^+$ Tregs were detected in both rTPO- and mock-treated mice, but their proportion was significantly greater in the rTPO-treated mice (Fig. 2), suggesting that short-term rTPO treatment promotes the peripheral induction of $Foxp3^+$ Tregs. We also measured the circulating TGF-β before and 2 weeks after rTPO or mock treatment. TGF-β levels were significantly increased after rTPO treatment (0.45 ± 0.09 vs 3.03 ± 4.69 pg/mL, $P < 0.05$), but not after mock treatment (0.58 ± 0.21 vs 0.41 ± 0.01 pg/mL). This effect is probably due to increased platelet and megakaryocyte counts, since treating ITP patients with eltrombopag results in increased circulating TGF-β levels in correlation with increased platelet numbers [14]. TGF-β is essential for maintenance of Treg function, and treatment with eltrombopag also improves Treg function in ITP patients [14]. Taken together, TGF-β released from platelets and megakaryocytes, which are expanded by treatment with thrombopoietic agents, may contribute to the differentiation of acquired $Foxp3^+$ Tregs and enhancement of their immunosuppressive functions. However, other mechanisms leading to immune tolerance such as exposure to high doses of antigen, an approach used to induce immune tolerance to exogenous factor VIII in hemophilia patients [15], also definitely play a role, since adaptive transfer of Tregs alone after onset of thrombocytopenia failed to increase platelet count in our mouse model [4].

In summary, short-term treatment with a thrombopoietic agent induced immune tolerance to platelet autoantigens in a mouse ITP model, suggesting that TPO-RAs may function in part by suppressing the immunopathogenic process of ITP. However, in ITP patients, sustained platelet recovery after discontinuation of TPO-RAs is much less common than in the animal model. Future studies will be required to identify factors controlling the immune tolerance induction in response to TPO-RAs in ITP patients.

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Conflict of interests The authors declare no conflict of interest.

References

1. Semple JW, Provan D. The immunopathogenesis of immune thrombocytopenia: T cells still take center-stage. Curr Opin Hematol. 2012;19:357–62.
2. Kuwana M, Kaburaki J, Ikeda Y. Autoreactive cells to platelet GPIIb-IIIa in immune thrombocytopenic purpura: role in production of anti-platelet autoantibody. J Clin Invest. 1998;02:1393–402.
3. Nishimoto T, Kuwana M. $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells in pathophysiology of immune thrombocytopenia. Semin Hematol. 2013;50:S43–9.
4. Nishimoto T, Satoh T, Takeuchi T, Ikeda Y, Kuwana M. Critical role of CD4(+)CD25(+) regulatory T cells in preventing murine autoantibody-mediated thrombocytopenia. Exp Hematol. 2012;40:279–89.
5. Nishimoto T, Satoh T, Simpson EK, Ni H, Kuwana M. Predominant autoantibody response to GPIb/IX in a regulatory T-cell-deficient mouse model for immune thrombocytopenia. J Thromb Haemost. 2013;11:369–72.
6. Kuter DJ. The biology of thrombopoietin and thrombopoietin receptor agonists. Int J Hematol. 2013;98:10–23.
7. Khellaf M, Michel M, Quittet P, et al. Romiplostim safety and efficacy for immune thrombocytopenia in clinical practice: 2-year results of 72 adults in a romiplostim compassionate-use program. Blood. 2011;118:4338–45.
8. Bussel JB, Buchanan GR, Nugent DJ, et al. A randomized, double-blind study of romiplostim to determine its safety and efficacy in children with immune thrombocytopenia. Blood. 2011;118:28–36.
9. Ghadaki B, Nazi I, Kelton JG, Arnold DM. Sustained remissions of immune thrombocytopenia associated with the use of thrombopoietin receptor agonists. Transfusion. 2013;53:2807–12.
10. Gómez-Almague D, Herrera-Rojas MA, Jaime-Pérez JC, et al. Eltrombopag and high-dose dexamethasone as frontline treatment of newly diagnosed immune thrombocytopenia in adults. Blood. 2014;123:3906–8.
11. Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y, Kawakami Y. T cells that are autoreactive to β2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. Arthritis Rheum. 2000;43:65–75.
12. Miyazaki H. Physiologic role of TPO in thrombopoiesis. Stem Cells. 1996;14:133–8.
13. Shibuya K, Kuwaki T, Tahara E, et al. Marked improvement of thrombocytopenia in a murine model of idiopathic thrombocytopenic purpura by pegylated recombinant human megakaryocyte growth and development factor. Exp Hematol. 2002;30:1185–92.
14. Bao W, Bussel JB, Heck S, et al. Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents. Blood. 2010;116:4639–45.
15. Reipert BM, van Helden PM, Schwarz HP, Hausl C. Mechanisms of action of immune tolerance induction against factor VIII in patients with congenital haemophilia A and factor VIII inhibitors. Br J Haematol. 2007;136:12–25.

血栓性血小板減少性紫斑病
thrombotic thrombocytopenic purpura（TTP）

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病態と診断

Ⓐ 病態

血栓性血小板減少性紫斑病（TTP）は全身の微小血管に血小板血栓が多発することによって脳，腎臓，心臓，消化管などの虚血性障害を起こす疾患で，消費性血小板減少と微小血管障害性溶血性貧血（microangiopathic hemolytic anemia：MAHA）を呈する．TTPの原因としてADAMTS13活性の低下が知られている．ADAMTS13は血管内皮から分泌されたvon Willebrand因子（VWF）を切断する酵素であって，未切断のVWFは血小板との結合活性がきわめて高いため血管内で血小板凝集塊が形成される．ADAMTS13活性低下は，*ADAMTS13*遺伝子異常（先天性TTP）もしくはADAMTS13に対する自己抗体（後天性TTP）によって引き起こされる．後天性TTPには明らかな原因のない特発性と，膠原病，膵炎，薬剤，移植などに続発する2次性TTPがある．それらのなかには自己抗体が検出されずにADAMTS13活性が保たれている症例があり，高サイトカイン血症を伴う全身炎症病態によるvWF増加が相対的ADAMTS13欠乏をきたしてTTPを発症している可能性，もしくはADAMTS13とは別の発症機序が想定される．ADAMTS13活性が著減しているTTPを定型TTPと定義すると，定型TTP以外は溶血性尿毒症症候群（HUS）と鑑別し難くなることが多く，血栓性微小血管障害症（thrombotic microangiopathy：TMA）という包括疾患名でよばれる．

Ⓑ 診断

血小板減少とMAHA（破砕赤血球を伴うクームス陰性溶血性貧血）があればTTPを疑う．同様の所見は播種性血管内凝固症候群（DIC）でもみられるが，TTPでは破砕赤血球が目立って溶血所見が激しいわりに，凝固線溶系異常が乏しく腎障害が軽度である．動揺する多彩な精神神経症状を見逃さない．ADAMTS13活性およびインヒビター（自己抗体）の測定は，治療で予後が大きく改善する定型TTPの確定診断に有用である（保険適用外）．

治療方針

TTPと臨床診断したならADAMTS13活性の測定結果を必ずしも待つ必要はなく，できるだけ早く血漿交換を開始する．たとえADAMTS13活性が低下していなくても血漿交換の適応はある．発症早期の死亡はすみやかな血漿交換によって防止可能である．ただし，造血幹細胞移植と悪性腫瘍に続発する2次性TTPの治療効果は乏しく，積極的な血漿交換は行わない．また，出血予防目的での血小板輸血は避ける（原則禁忌）．抗血小板薬併用の有用性は明らかでなく，少なくとも急性期は必要でない．

Ⓐ 血漿交換療法

血漿交換開始3日間は循環血漿量の1.5倍で連日行い，その後は循環血漿量の1倍に減量する．病初期に血小板数がやや増加したからといって血漿交換を1日でも休むと，病態が急速に悪化することがある．治療開始7－10日後に血小板数10万/μL未満なら週5回以上，10万/μL以上なら週3回で継続する．その後，血小板数15万/μL以上が持続するなら中止する．ただし，日本では血漿交換を週3回，3か月までとする保険上の制限が記されている．

Ⓡ 処方例

新鮮凍結血漿－LR（FFP－LR）　循環血漿量（40 mL/kg）の1.5倍量を置換液として1日1回，3日間，その後は1倍量で1日1回連日

FFP製剤の単位表記は廃止された．FFP－LR 480（旧5単位成分採血製剤）＝480 mL，FFP－LR 240（旧2単位製剤）＝240 mL．患者体重60 kgの場合，3,600 mLのFFPで開始になるが，この量は120 mL＝1単位とすれば30単位に相当する．

Ⓑ 血漿輸注療法

血漿交換がすぐに開始できないときはとりあえず血漿輸注を行うが，できるだけ早く血漿交換に切り替える．なお，先天性TTPでは血漿交換不要で，血小板数とHbが正常に保てるような間隔で血漿輸注（ADAMTS13の補充）を行う．したがって投与間隔は症例ごとに異なり，2週間ごとに輸注が必要な症例から，日常に輸注を必要とせず感染や妊娠

などのリスク要因が負荷されて血小板減少が出現したときにのみ輸注する症例までである.

Rp 処方例

新鮮凍結血漿-LR (FFP-LR)　1回10-15 mL/kgを輸注

◎免疫抑制療法

後天性TTPは自己免疫疾患の側面があるので,血漿交換と併用して用いられる.初回治療例では処方例1)を用いる.血漿交換開始1週後に症状または所見の改善がないかADAMTS13インヒビター値が有意に上昇してくる例では2)を追加し,それでも無効なら3)または4)を投与する.

Rp 処方例　下記1)を用いる.難治例は2)　3)　4)のいずれかを追加する.

1) ソル・メドロール注　1回1g　1日1回　血漿交換直後に点滴静注　3日間 保外　その後プレドニン錠 (5mg)　0.5-1mg/kg/日　経口にして漸減 保外
2) リツキサン注　1回375mg/m²　週1回　血漿交換直後に点滴静注　4回 保外
3) オンコビン注　初回1-2mg　静注　1週後1mg追加 保外
4) ネオーラルカプセル (50mg)　4-6mg/kg　分2 保外 47

1 先天性および後天性血管障害による出血

羽藤高明

疾患の概説

血管内皮あるいは血管壁に異常があると，紫斑をはじめとする出血症状が生じる．先天性疾患としては血管新生あるいは血管壁構成成分の異常症が多く，後天性疾患としては血管炎が多い．本項では臨床的に重要な血管障害性紫斑病について概説し，皮膚・血管老化に伴う老人性紫斑病，原因不明の若年者紫斑に対して診断される単純性紫斑病，ステロイド薬の長期連用によってコラーゲンが分解されることによるステロイド紫斑病は日常臨床でしばしば遭遇する疾患であるが割愛する．

a 先天性血管障害

❶遺伝性出血性毛細血管拡張症（Rendu-Osler-Weber 病）

臨床所見として，①反復性鼻出血，②特徴的部位（口唇，口腔，指尖，鼻腔）にみられる毛細血管拡張（点状・斑状赤色小隆起），③内臓の血管病変：消化管の毛細血管拡張，肺，脳，肝の動静脈奇形（arteriovenous malformation：AVM），④家族内発症（常染色体優性遺伝）があり，これら4項目のうち3つ以上あれば診断は確定する．原因遺伝子として *ENG*/endoglin と *ACVRL1*/ALK1 が同定されており，また若年性ポリポーシスを伴う病型では *MADH4*/Smad4 の異常が同定されている．いずれの遺伝子も TGFβ スーパーファミリーに属するシグナル伝達分子をコードしている．これらの異常によって血管新生因子と血管新生抑制因子のアンバランスが生じ，この状態に炎症などの刺激（second hit）が加わると異常な血管新生が起こって本症の血管病変が生じるとされている．したがって，本症でみられる血管病変・症状は年齢とともに発現してくるのが特徴である．予後はこれら AVM に伴う合併症に左右される．直接死因となるのは肺出血もしくは脳出血であるが，大きな AVM がなく重篤な合併症がない症例の生命予後は一般に良好である．

❷Ehlers-Danlos 症候群

皮膚の過伸展，関節可動域過大，易出血性が特徴である．皮膚は菲薄化して皮下の血管が透けて見えるほどのこともあり，微少な外傷で紫斑を生じやすい．症状の重篤度やその他の症状・所見の存在，遺伝形式によって6病型に分けられている．典型例（classical type）は“ゴム人間”と呼ばれるような皮膚・関節の過伸展を示し，関節脱臼や鼠径・臍ヘルニア，僧帽弁逸脱を合併しやすい．予後が悪いのは血管型（vascular type）と呼ばれる病型で，皮膚・関節症状は目立たないが動脈壁コラーゲン組織の脆弱性のため動脈破裂を起こして突然死することが多く，平均余命は48歳である．また自然気胸，腸管穿孔や妊娠に伴う子宮破裂をきたしやすい．原因遺伝子として classical type ではコラーゲン Type Ⅴの α_1，α_2鎖をコードしている *COL5A1*，*COL5A2* が知られており，血管型ではコラーゲン Type Ⅲの α_1鎖をコードしている *COL3A1* が同定されていて，いずれも常

TOPICS

遺伝性出血性毛細血管拡張症に対する Bevacizumab 療法

遺伝性出血性毛細血管拡張症は血管新生の異常を基盤とする疾患であるが，本症に対して抗 VEGF 作用を有する bevacizumab（アバスチン）が劇的に奏効したとの症例報告が相次ぎ，それを受けて第Ⅱ相臨床試験が行われた．肝 AVM による高拍出性心不全をきたしている24症例に bevacizumab を投与したところ，心拍出量の正常化が3例，改善が17例にみられた．さらに鼻出血は80％減少し，患者の QOL が明らかに改善された．高血圧以外に主な有害事象はなかった．

Dupis-Girod S et al：Bevacizumab in patients with hereditary hereditary hemorrhagic telangiectasia and severe hepatic vascular malformations and high cardiac output. JAMA 307：948-955, 2012

染色体優性遺伝形式をとる.

b 後天性血管障害

❶Schönlein-Henoch 紫斑病

紫斑, 関節痛, 消化管症状（腹痛, 下血）, 糸球体腎炎を呈する疾患でアレルギー性紫斑病とも呼ばれる. 4〜7歳の小児に多いが成人例もある. 誘因として上気道感染, 薬剤, 食物, 虫刺, 予防接種などがあげられているが, 本態はIgAを主とする免疫複合体の組織沈着によって引き起こされる血管炎である. 紫斑は丘疹性であることが多く, 四肢, 特に下腿伸側に対称性に出現し, 殿部にまで及ぶことはあるが躯幹には少ない. 予後は一般に良好で通常3ヵ月以内に自然治癒するが, 再燃を繰り返すこともある. 一部の患者は腎炎の遷延化または急速進行性腎炎の経過をとることがある.

❷クリオグロブリン血症

クリオグロブリンは低温で可逆性に凝固する免疫グロブリンであり, さまざまな基礎疾患のもとに出現し, 紫斑, 関節痛, 末梢神経炎, 糸球体腎炎を引き起こす. 紫斑は本症で最も頻度の高い症状であり, 寒冷曝露を誘因として四肢露出部位に生じ, 色素沈着を残して消退するが再発が多い. 手指末端の出血性壊死, 下腿皮膚潰瘍, 爪下出血がみられることもある. また, 脱力感や対称性の関節痛を伴うことが多く, 下肢の末梢神経炎による運動・感覚障害がみられることもある. 本態はクリオグロブリンによって誘発される血管炎であり, Schönlein-Henoch 紫斑病と症状は類似するが, 末梢神経障害があればクリオグロブリン血症である可能性が高い. クリオグロブリンがM蛋白を含んでいる場合は基礎疾患としてB細胞腫瘍が示唆され, その検索が重要である. 一方, ポリクローナルの場合は膠原病もしくはC型肝炎ウイルス（HCV）感染が基盤にあることが多く, HCVに対する異常免疫反応が原因と考えられている.

治療のための診断・検査

a 先天性血管障害

❶遺伝性出血性毛細血管拡張症（Rendu-Osler-Weber 病）

血液検査では鼻出血や消化管出血に起因する鉄欠乏性貧血がみられる. AVMの検索は診断に有用であるばかりでなく, 治療方針を決定するのに必須である. 肺AVMはコントラスト心エコーで最も感度よく検出できるが, CTでも十分検出可能である. 脳AVMはMRIによって評価し, 肝AVMにはドップラー超音波検査が適している. 消化管病変は胃・十二指腸に多く, 上部消化管内視鏡検査で検索する.

❷Ehlers-Danlos 症候群

突然死の家族歴と自然気胸や腸管穿孔の既往から血管型が疑われたら, CTなどで大動脈瘤や動脈解離所見の有無を検索する. 血液検査は正常であり, 血管型の臨床診断は困難なので遺伝子診断に頼るしかない面がある.

b 後天性血管障害

❶Schönlein-Henoch 紫斑病

予後を左右する糸球体腎炎の検索は重要で, 紫斑出現1ヵ月後までは尿検査をfollowする. 腎生検が確定診断に使われることは少なく, 腎炎の遷延化や腎障害が重篤な場合にのみ考慮される. 腹痛・下血は消化管壁の血管炎による浮腫と出血に起因しており, 内視鏡検査では, 発赤, 浮腫, びらん, 潰瘍などの所見がみられる.

❷クリオグロブリン血症

クリオグロブリン測定に関しては採血直後から血清分離まで37℃に保つことが大切である. 基礎疾患の検索が重要で, 免疫電気泳動, 骨髄穿刺, 画像診断などを行ってB細胞腫瘍を検索する. また, HCV抗体, HCV-RNAの検査を行う.

治療の一般方針

a 先天性血管障害

❶遺伝性出血性毛細血管拡張症（Rendu-Osler-Weber 病）

鼻出血には耳鼻科的局所処置を行うが難治性である. 閉経前女性ではエストロゲン・プロゲステロン合剤（ルナベル）が奏効するとの報告があるが, 閉経後女性では長期服用による子宮がん・乳がん発症と血栓症誘発のリスクが高まるため推奨されない. 男性にはアンドロゲン製剤（ボンゾール）が使用される. 抗エストロゲン薬である