日間，以後プレドニゾロンを用いて漸減し，First line 治療の副腎皮質ステロイド療法を実施することが一般的である。

急性 ITP に使用され効果を認めているが難治性 ITP にも用いられ，約 80％の症例で 10 万/μl 以上に血小板数の増加が認められている。

反応は 3 日目くらいより現れるが，一過性である。

おわりに

今回本邦の実情に即した ITP 治療の参照ガイドを作成した。今後多くの症例で検討していただき，有用性，妥当性についてご意見をいただくことを願っている。また今後新たな治療薬剤の開発やエビデンスに基づいた治療法の研究により，治療の参照ガイドを適宜改訂して行く予定である。

謝　辞

この ITP 治療の参照ガイドは厚生労働省難治性疾患克服事業「血液凝固異常症調査研究班」(研究代表者 村田満) のもとで作成された。本研究班からの ITP アンケート調査に協力頂いた全国の日本血液学会血液専門医の方々，また研究協力者である三重大学 和田英夫先生，名古屋大学 小嶋哲人先生，関西医科大学病院 野村昌作先生，西神戸医療センター 高蓋寿朗先生から多大な協力を頂きここに深甚なる謝意を捧げます。(本研究の一部は厚生労働省科学研究費補助金 難治性疾患克服研究事業 血液凝固異常症に関する調査研究からの補助を受けた)

著者の COI (conflicts of interest) 開示：宮川義隆：報酬 (エーザイ株式会社，協和発酵キリン株式会社)，講演料 (協和発酵キリン株式会社，グラクソ・スミスクライン株式会社)，冨山佳昭；報酬 (グラクソ・スミスクライン株式会社)，講演料 (グラクソ・スミスクライン株式会社)，村田満；報酬 (グラクソ・スミスクライン株式会社，第一三共株式会社，ファイザー株式会社)，藤村欣吾；報酬 (グラクソ・スミスクライン株式会社，協和発酵キリン株式会社)，その他 藤村欣吾，宮川義隆，倉田義之，桑名正隆，冨山佳昭，村田満 (厚生労働科学研究治験推進研究事業)

文　献

1) 野村武夫. 特発性血小板減少性紫斑病 診断・治療の手引き. 厚生省特定疾患特発性造血障害調査研究班 (班長 野村武夫). 昭和 63 年度研究業績報告書. 1989; 付表 3: 38-60.
2) 野村武夫, 厨信一郎, 蔵本淳, 前川正. 特発性血小板減少性紫斑病の治療に関する多施設共同プロスペクティブ研究 特発性造血障害調査研究班 (班長 野村武夫). 昭和 63 年度研究業績報告書. 1989; 136-137.
3) Cohen YC, Djulbegovic B, Shamai-Lubovitz O, Mozes B. The bleeding risk and natural history of idiopathic thrombocytopenic purpura in patients with persistent low platelet counts. Arch Intern Med. 2000; **160**: 1630-1638.
4) Portielje JE, Westendorp RG, Kluin-Nelemans HC, Brand A. Morbidity and mortality in adults with idiopathic thrombocytopenic purpura. Blood. 2001; **97**: 2549-2554.
5) Frederiksen H, Schmidt K. The incidence of idiopathic thrombocytopenic purpura in adults incases with age. Blood. 1999; **94**: 909-913.
6) Kurata Y, Fujimura K, Kuwana M, Tomiyama Y, Murata M. Epidemiology of primary immune thrombocytopenia in children and adults in Japan: a population-based study and literature review. Int J Hematol. 2011; **93**: 329-335.
7) Gasbarrini A, Franceschi F, Tartaglione R, Landolfi R, Pola P, Gasbarrini G. Regression of autoimmune thrombocytopenia after eradication of Helicobacter pylori. Lancet. 1998; **352**: 878.
8) Emillia G, Longo G, Luppi M, et al. Helicobacter pylori eradication can induce platelet recovery in idiopathic thrombocytopenic purpura. Blood. 2001; **97**: 812-814.
9) Fujimura K, Kuwana M, Kurata Y, et al. Is eradication therapy useful as the first line of treatment in Helicobacter pylori-positive idiopathic thrombocytopenic purpura? Analysis of 207 eradicated chronic ITP cases in Japan. Int J Hematol. 2005; **81**: 162-168.
10) 桑名正隆, 池田康夫. ITP における *Helicobacter pylori* 除菌効果発現機序の解析 (主任研究者 池田康夫). 厚生労働省科学研究費補助金 難治性疾患克服研究事業 血液凝固異常症に関する調査研究. 平成 18 年度報告書. 2007. **3**; 83-88.
11) Kuwana M, Kurata Y, Fujimura K, et al. Preliminary laboratory based diagnostic criteria for immune thrombocytopenic purpura: evaluation by multi-center prospective study. J Thromb Haemost. 2006; **4**: 1936-1943.
12) McMillan R, Durette C. Long-term outcomes in adults with chronic ITP after splenectomy failure. Blood. 2004; **104**: 956-960.
13) Cines DB, Bussel JB. How I treat idiopathic thrombocytopenic purpura (ITP). Blood. 2005; **106**: 2244-2251.
14) George JN. Management of patients with refractory immune thrombocytopenic purpura. J Thromb Haemost. 2006; **4**: 1664-1672.
15) George JN, Woolf SH, Raskob GE, et al. Idiopathic thrombocytopenic Purpura: a practice guideline developed by explicit methods for the American Society of Hematology. Blood. 1996; **88**: 3-40.
16) British Committee for Standards in Haematology General Haematology Task Force. Guidelines for the investigation and management of idiopathic thrombocytopenic purpura in adults, children and in pregnancy. Br J Haematol. 2003; **120**: 574-596.
17) Provan D, Stasi R, Newland AC, et al. International consensus report on the investigation and management of primary im-

mune thrombocytopenia. Blood. 2010; **115**: 168-186.

18) Neunert C, Lim w, Crowther M, et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood. 2011; **117**: 4190-4207.

19) 藤村欣吾. 免疫性血小板減少性紫斑病（ITP）における治療ガイドライン（案）の提案―ヘリコバクタピロリ菌除菌療法の成績を踏まえて（主任研究者 池田康夫）. 厚生労働科学研究費補助金 難治性疾患克服研究事業 血液凝固異常症に関する調査研究. 平成16年度報告書. 2005. **3**; 53-69.

20) 日本ヘリコバクター学会ガイドライン2009. 日本ヘリコバクター学会 "*H. pylori* 感染の診断と治療のガイドライン" 2009改訂版 Helicobacter Research. 2009; **12**: 386-460.

21) Stasi R, Sarpatwari A, Segal JB, et al. Effects of eradication of Helicobacter pylori infection in patients with immune thrombocytopenic purpura: a systematic review. Blood. 2009; **113**: 1231-1240.

22) Kojouri K, Vesely SK, Terrell DR, George JN. Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, production of response, and surgical complications. Blood. 2004; **104**: 2623-2634.

23) 蔵本淳, 小田健司, 川野宏則, ほか. 多施設共同研究による摘脾療法の遠隔成績 厚生省特定疾患 特発性造血障害調査研究班（班長 野村武夫）. 平成4年度研究業績報告書. 1993; **3**: 130-133.

24) 藤村欣吾. ―難治性ITPの実態調査―平成18年度アンケート調査の報告―（主任研究者 池田 康夫）. 厚生労働科学研究費補助金 難治性疾患克服研究事業 血液凝固異常症に関する調査研究. 平成18年度報告書. 2007. **3**; 73-82.

25) Bussel JB, Kuter DJ, George JN, et al. AMG 531, a thrombopoiesis-stimulating protein, for chronic ITP. N Engl J Med. 2006; **355**: 1672-1681.

26) Jenkins JM, Williams D, Deng Y, et al. Phase 1 clinical study of eltrombopag, an oral, nonpeptide thrombopoietin receptor agonist. Blood. 2007; **109**: 4739-4741.

27) Bussel JB, Cheng G, Saleh MN, et al. Eltrombopag for the treatment of chronic idiopathic thrombocytopenic purpura. N Engl J Med. 2007; **357**: 2237-2247.

28) Kuter DJ, Bussel JB, Lyons RM, et al. Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. Lancet. 2008; **371**: 395-403.

29) Maloisel F, Andrès E, Zimmer J, et al. Danazol therapy in patients with chronic idiopathic thrombocytopenic purpura: long-term results. Am J Med. 2004; **116**: 590-594.

30) 野村武夫, 前川正, 檀和夫. 特発性血小板減少性紫斑病に対するダナゾール療法―多施設共同研究―特発性造血障害調査研究班（班長 前川正）. 昭和59年度研究業績報告書. 1985; **3**: 344-350.

31) McMillan R. Therapy for adults with refractory chronic immune thrombocytopenic purpura. Ann Intern Med. 1997; **126**: 307-314.

32) Cooper N, Stasi R, Cunningham-Rundles S, et al. The efficacy and safety of B-cell depletion with anti-CD20 monoclonal antibody in adults with chronic immune thrombocytopenic purpura. Br J Haematol. 2004; **125**: 232-239.

33) Arnold DM, Dentali F, Crowther MA, et al. Systematic review: efficacy and safety of rituximab for adults with idiopathic thrombocytopenic purpura. Ann Intern Med. 2007; **146**: 25-33.

34) Peñalver FJ, Jiménez-Yuste V, Almagro M, et al. Rituximab in the management of chronic immune thrombocytopenic purpura: an effective and safe therapeutic alternative in refractory patients. Ann Hematol. 2006; **85**: 400-406.

35) Godeau B, Porcher R, Fain O, et al. Rituximab efficacy and safety in adult splenectomy candidates with chronic immune thrombocytopenic purpura: results of a prospective multicenter phase 2 study. Blood. 2008; **112**: 999-1004.

36) Carson KR, Evens AM, Richey EA, et al. Progressive multifocal leukoencephalopathy after rituximab therapy in HIV-negative patients: a report of 57 cases from the Research on Adverse Drug Events and Reports project. Blood. 2009; **113**: 4834-4840.

37) Emillia G, Morselli M, Luppi M, et al. Long-term salvage therapy with cyclosporin A in refractory idiopathic thrombocytopenic purpura. Blood. 2002; **99**: 1482-1485.

38) Vesely SK, Perdue JJ, Rizvi MA, Terrell DR, George JN. Management of adult patients with persistent idiopathic thrombocytopenic purpura following splenectomy: a systematic review. Ann Intern Med. 2004; **140**: 112-120.

39) Anderson JC. Response of resistant idiopathic thrombocytopenic purpura to pulsed high-dose dexamethasone therapy. N Engl J Med. 1994; **330**: 1560-1564.

40) Cheng Y, Wong SM, Soo YO, et al. Initial treatment of immune thrombocytopenic purpura with high-dose dexamethasone. N Engl J Med. 2003; **349**: 831-836.

41) 野村武夫, 前川正. 特発性血小板減少性紫斑病のビンカアルカロイド緩速点滴静注療法に関する多施設共同パイロット研究 特発性造血障害調査研究班（班長 前川正）. 昭和62年度研究業績報告書. 1988; **3**: 188-189.

42) Bussel JB, Pham LC. Intravenous treatment with gammaglobulin in adults with immune thrombocytopenic purpura: review of the literature. Vox Sang. 1987; **52**: 206-211.

Downloaded from ard.bmj.com on April 5, 2012 - Published by group.bmj.com

CONCISE REPORT

Quantification of circulating endothelial progenitor cells in systemic sclerosis: a direct comparison of protocols

Masataka Kuwana, Yuka Okazaki

▸ Additional data are published online only. To view the files please visit the journal online (http://ard.bmj.com)

Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

Correspondence to
Masataka Kuwana, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; kuwanam@z5.keio.jp

Received 5 September 2011
Accepted 11 December 2011
Published Online First
17 January 2012

ABSTRACT

Background It has been proposed that dysfunctional endothelial progenitor cells (EPCs) play a role in pathogenic vasculopathy in systemic sclerosis (SSc). However, there is some debate as to whether the EPC count is reduced in SSc. The European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) group recently proposed recommendations for evaluating EPCs.

Objective To validate the proposed EUSTAR recommendations by a side-by-side comparison of methods for quantifying EPCs.

Methods Peripheral blood samples were obtained from 11 patients with SSc and 11 age-matched healthy controls. EPCs were simultaneously quantified by two methods: flow cytometry combined with immunomagnetic CD34+ cell enrichment or rosette-based lineage-negative (Lin−) cell enrichment. EPCs, defined as CD34+CD133+VEGFR2+ cells, were counted with and without fluorosphere calibration.

Results EPC counts measured with fluorosphere calibration correlated well with each other, regardless of the enrichment procedure used. In contrast, EPC counts from protocols that did not use fluorospheres correlated poorly with results from other protocols.

Conclusions The EUSTAR recommendations are valid when they are combined with fluorosphere calibration.

INTRODUCTION

In adults, new blood vessels are formed by at least two mechanisms: endothelial sprouting from pre-existing endothelial cells (angiogenesis) and the peripheral recruitment of bone marrow-derived endothelial progenitor cells (EPCs) (vasculogenesis). Over the last decade, EPCs have emerged as crucial regulators of vascular healing and remodelling, homing in on injury sites and working in concert with mature endothelial cells.[1] The role of EPCs in the vascular pathogenesis of connective tissue disease has therefore attracted considerable attention, both as potential biomarkers for vascular manifestations and as novel therapeutic targets. It has been shown that EPC counts are altered in patients with systemic sclerosis (SSc), rheumatoid arthritis, systemic lupus erythematosus and vasculitis.[2] However, research findings have varied. Since our report of reduced EPC counts in patients with SSc,[3] some researchers subsequently confirmed our finding[4 5] but others found an increased number of EPCs in patients with SSc.[6–9] These contradictory results may result from differences in the protocols used for quantifying EPCs. In an effort to standardise EPC research, the European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) recently proposed recommendations on EPC detection methods.[10] However, these recommendations have not yet been validated. In this study we examined the reproducibility of the different EPC quantification protocols that would conform to EUSTAR recommendations by examining samples from patients with SSc and healthy controls.

METHODS

Patients and controls

The study included 11 women with SSc who were followed at Keio University Hospital. All patients fulfilled the American College of Rheumatology preliminary classification criteria for SSc.[11] Patients taking an immunosuppressant, statin or >10 mg/day prednisolone were excluded. Eleven healthy women age-matched with the patients with SSc were recruited from the hospital staff. All subjects were free of diabetes, hypertension or dyslipidaemia and none of the subjects smoked. The age at evaluation was similar between the patients with SSc and the healthy controls (mean±SD 60.5±14.7 years and 56.8±14.6 years). Six were classified as having diffuse cutaneous SSc. The disease duration from the onset of non-Raynaud's symptoms ranged from 12 to 262 months.

EPC quantification

Heparinised peripheral blood samples (60 ml) were taken from a forearm vein while the subject was at rest. The samples were taken in the morning and immediately transported to the laboratory where each sample was divided equally into two tubes. EPCs were quantified by flow cytometric analysis of partially enriched progenitor cells according to two methods, as published previously[3 8]: CD34+ cells by a magnetic-activated cell sorter (MACS) technique (MACS method)[3] or lineage-negative (Lin$^-$) cells by a rosette-based technique (rosette method).[8] The viability marker 7-amino actinomycin D (7AAD) was used in a multiparameter flow cytometer. These methods conform to the EUSTAR recommendations for flow cytometry (see online supplement).[10] EPCs were identified by the co-expression of CD34, CD133 and vascular endothelial growth factor receptor type 2 (VEGFR2) and were expressed as the number per 10^6 CD34+ or Lin− cells, counted based on the acquisitioned events on a flow cytometer. In addition, the absolute number of EPCs in 1 ml peripheral blood was calculated using FlowCount polystyrene fluorospheres as an internal calibrator.

Downloaded from ard.bmj.com on April 5, 2012 - Published by group.bmj.com

Table 1 Correlation coefficient between different methods of EPC quantification in 11 patients with SSc and 11 healthy controls

Method for EPC quantification	MACS/7AAD− /fluorosphere+ (/1 ml PB)	MACS/7AAD+ /fluorosphere+ (/1 ml PB)	MACS/7AAD+ /fluorosphere− (/10⁶ CD34+ cells)	Rosette/7AAD+ /fluorosphere+ (/1 ml PB)	Rosette/7AAD+ /fluorosphere− (/10⁶ Lin− cells)
MACS/7AAD−/fluorosphere+ (/1 ml PB)		0.95†	0.17	0.73†	0.24
MACS/7AAD+/fluorosphere+ (/1 ml PB)	–		0.28	0.81†	0.26
MACS/7AAD+/fluorosphere− (/10⁶ CD34+ cells)	–	–		0.30	0.11
Rosette/7AAD+/fluorosphere+ (/1 ml PB)	–	–	–		0.40
Rosette/7AAD+/fluorosphere− (/10⁶ Lin− cells)	–	–	–	–	

†p<0.01.
EPC, endothelial progenitor cell; Lin−, lineage-negative; MACS, magnetic-activated cell sorter; PB, peripheral blood; SSc, systemic sclerosis.

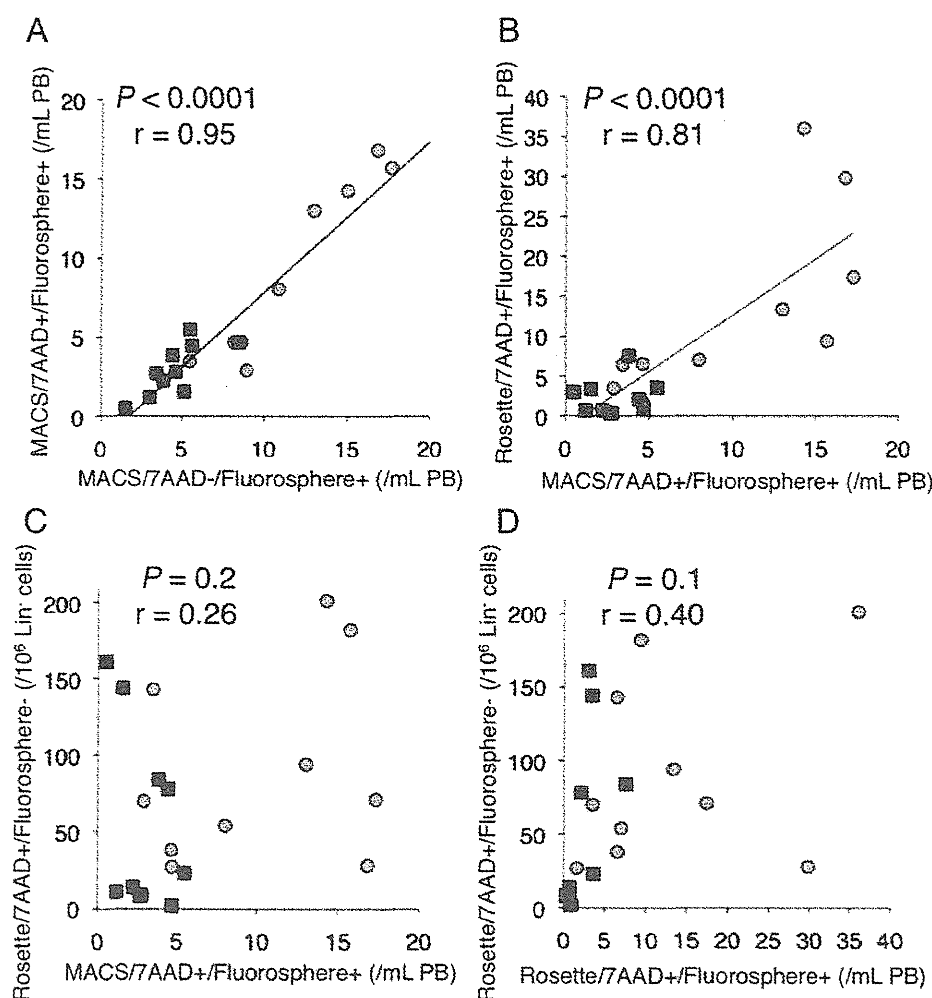

Figure 1 Correlations between endothelial progenitor cells (EPC) counts measured simultaneously by different protocols in 11 patients with systemic sclerosis (SSc, squares) and 11 healthy controls (circles). (A) Correlation between the magnetic-activated cell sorter (MACS) method with fluorosphere calibration, with and without 7-amino actinomycin D (7AAD) staining. (B) Correlation between the MACS and rosette methods with 7AAD staining and fluorosphere calibration. (C) Correlation between the MACS method with 7AAD staining and fluorosphere calibration, and the rosette method with 7AAD staining and no fluorosphere calibration. (D) Correlation between the rosette method with 7AAD staining, with and without fluorosphere calibration. PB, peripheral blood.

All procedures were performed by an experienced flow cytometry operator who was blinded to the sample identity.

Analysis of contaminating cell types in the Lin− cell fraction

The enriched Lin⁻ cell fraction was incubated with a combination of fluorescently labelled monoclonal antibodies anti-CD3, anti-CD14, anti-CD15, anti-CD41, antiglycophorin A (Beckman-Coulter, Fullerton, CA, USA) and anti-CD19 (BD Biosciences, San Diego, CA, USA). A flow cytometer was used to detect fluorescent cell staining. Dead cells were excluded by scatter analysis and by 7AAD staining.

Statistical analysis

The correlation coefficient (r) was determined using a single regression model. Comparisons between two groups were tested for statistical significance using the Mann–Whitney U test.

Downloaded from ard.bmj.com on April 5, 2012 - Published by group.bmj.com

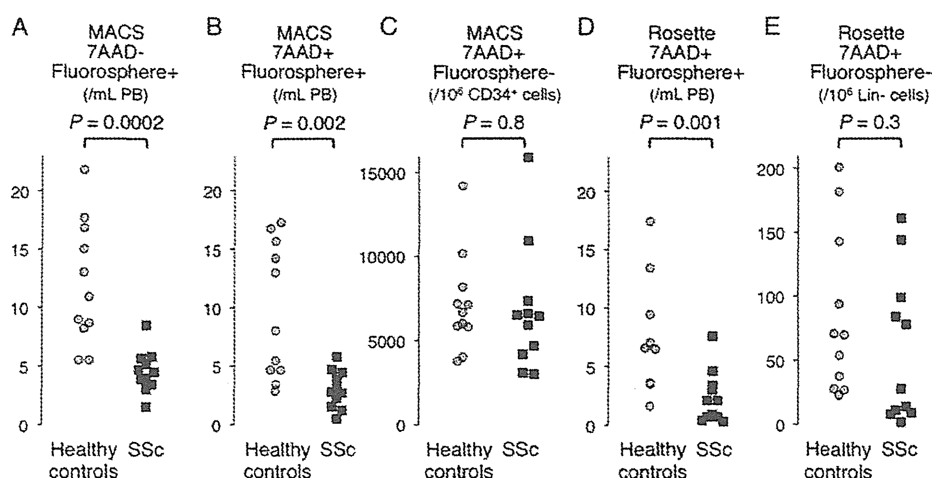

Figure 2 Comparison of endothelial progenitor cells (EPC) counts measured in 11 patients with systemic sclerosis (SSc) and 11 healthy controls using five different protocols. (A) Magnetic-activated cell sorter (MACS) method without 7-amino actinomycin D (7AAD) staining and with fluorosphere calibration. (B) MACS method with 7AAD staining and fluorosphere calibration. (C) MACS method with 7AAD staining but without fluorosphere calibration. (D) Rosette method with 7AAD staining and fluorosphere calibration. (E) Rosette method with 7AAD staining but without fluorosphere calibration. PB, peripheral blood, HC, healthy controls.

RESULTS

Before beginning data collection we analysed more than 100 samples from patients with SSc and healthy individuals as a practice session. The coefficient of variation for four repeated measures of EPCs on five samples was 9–18% and 19–31% for the original MACS and rosette methods, respectively. Representative flow cytometric analyses using different protocols are shown in figures S1–3 in the online supplement. Nearly all the EPCs detected by these protocols were confirmed to be negative for CD14 but weakly positive for CD45.

EPCs were measured simultaneously in 11 patients with SSc and 11 healthy controls using five different protocols: (1) MACS method without 7AAD staining but with fluorosphere calibration (only method that did not conform to EUSTAR recommendations); (2) MACS method with both 7AAD staining and fluorosphere calibration; (3) MACS method with 7AAD staining but without fluorosphere calibration; (4) rosette method with both 7AAD staining and fluorosphere calibration; and (5) rosette method with 7AAD staining but without fluorosphere calibration. The correlation coefficients between the EPC counts obtained by these protocols are summarised in table 1. EPC counts obtained by the MACS method with and without 7AAD staining correlated well when fluorospheres were used (figure 1A; r=0.95, p<0.0001). Interestingly, there was a statistically significant correlation between EPC counts obtained by the MACS and rosette methods when combined with fluorosphere calibration (figure 1B; r=0.81, p<0.0001). In contrast, protocols that did not use fluorosphere calibration correlated poorly with other protocols, regardless of the method of progenitor cell enrichment used (figure 1C,D).

We further examined the purity of CD34+ or Lin− cells in the enriched fractions. CD34+ cells in the enriched fraction ranged from 4% to 49% after viable cells were selected by gating with scatter analysis and 7AAD staining. To evaluate the contaminating cells in the Lin⁻ cell fractions, multiparameter flow cytometry was performed using the gate setting used for EPC measurement (see figure S4 in online supplement). While CD3+ T cells, CD14+ monocytes, CD15+ granulocyte/monocytes and CD19+ B cells accounted for <6% of the cells in the Lin− fraction, the predominant cell populations consisted of CD41+ platelets and glycophorin A+ erythrocytes. When all the cells with lineage markers were excluded, the percentage of Lin− cells in the enriched fraction ranged from 8% to 28%.

When EPC counts between patients with SSc and healthy controls were compared (figure 2), protocols using fluorosphere quantification consistently detected reduced numbers of EPCs in SSc. In contrast, protocols that did not use fluorospheres did not find any difference between the two groups.

DISCUSSION

This is the first study to directly compare protocols for quantifying EPCs. Our findings indicate that the EUSTAR recommendations are valid when combined with an accurate quantification technique such as the use of fluorospheres as an internal calibrator. In previous studies EPCs were quantified using a variety of strategies and were expressed as a proportion (%) in mononuclear cells[4 7 9] or the absolute number in peripheral blood[3 5 6] or in 10^6 Lin− cells,[8] but our results indicate that the quantification strategy strongly affects the consistency of the results. The inter-method concordance was very poor when CD34+ or Lin− cell counts, based on acquisitioned events by a flow cytometer, were used as a reference. This is probably because CD34+ or Lin− cells are rare cell populations that comprise less than 1% of circulating mononuclear cells. The purity of the enriched fractions varied greatly between samples even when an intensive gating protocol was used. Under these circumstances, introducing a fluorosphere technique significantly and substantially improved the reproducibility of the results. Thus, incorporating this critical issue into the EUSTAR recommendations should allow better comparison between studies.

In this study we observed a reduced number of EPCs in patients with SSc. However, the number of subjects was too small to draw a conclusion. To answer the central question whether EPCs are increased or reduced in patients with SSc, large-scale studies that conform to EUSTAR recommendations and use an accurate calibration protocol are needed. These studies should include patients with other connective tissue

Downloaded from ard.bmj.com on April 5, 2012 - Published by group.bmj.com

diseases and should analyse potential correlations between EPC counts and clinical features of SSc.

The definition of EPCs is currently much debated.[12–14] Cells originally identified as EPCs in various assays are now known to be heterogeneous, and nearly all of the cells are members of the haematopoietic lineage and display pro-angiogenic properties. Non-haematopoietic circulating cells with a clonal proliferative potential and postnatal vasculogenic activity, termed endothelial colony-forming cells, are considered true EPCs, but these cells are detected only in long-term culture and are presumed to be very rare (<1 per 10^6 circulating mononuclear cells).[12] This makes it challenging to identify EPCs by flow cytometry. Nevertheless, CD34+CD133+VEGFR2+ cells have become widely used as a means of measuring putative circulating EPCs,[15] although a direct link between CD34+CD133+VEGFR2+ cells and the 'true EPCs' detected in culture is still missing. In fact, the majority of CD34+CD133+VEGFR2+ cells detected as EPCs in this study were apparently CD45+ haematopoietic progenitors. Because several different types of blood cells have been implicated as EPCs, further study is required to determine the exact role of each of these cell types in the pathogenic processes of SSc.

Funding Research grant on intractable diseases by the Japanese Ministry of Health, Labour, and Welfare; and the Japanese Ministry of Education, Science, Sports and Culture.

Ethics approval This study was approved by the relevant ethical committees.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Urbich C**, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 2004;**95**:343–53.
2. **Distler JH**, Beyer C, Schett G, *et al.* Endothelial progenitor cells: novel players in the pathogenesis of rheumatic diseases. *Arthritis Rheum* 2009;**60**:3168–79.
3. **Kuwana M**, Okazaki Y, Yasuoka H, *et al.* Defective vasculogenesis in systemic sclerosis. *Lancet* 2004;**364**:603–10.
4. **Zhu S**, Evans S, Yan B, *et al.* Transcriptional regulation of Bim by FOXO3a and Akt mediates scleroderma serum-induced apoptosis in endothelial progenitor cells. *Circulation* 2008;**118**:2156–65.
5. **Mok MY**, Yiu KH, Wong CY, *et al.* Low circulating level of CD133+KDR+cells in patients with systemic sclerosis. *Clin Exp Rheumatol* 2010;**28**(5 Suppl 62):S19–25.
6. **Del Papa N**, Quirici N, Soligo D, *et al.* Bone marrow endothelial progenitors are defective in systemic sclerosis. *Arthritis Rheum* 2006;**54**:2605–15.
7. **Allanore Y**, Batteux F, Avouac J, *et al.* Levels of circulating endothelial progenitor cells in systemic sclerosis. *Clin Exp Rheumatol* 2007;**25**:60–6.
8. **Avouac J**, Juin F, Wipff J, *et al.* Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. *Ann Rheum Dis* 2008;**67**:1455–60.
9. **Nevskaya T**, Bykovskaia S, Lyssuk E, *et al.* Circulating endothelial progenitor cells in systemic sclerosis: relation to impaired angiogenesis and cardiovascular manifestations. *Clin Exp Rheumatol* 2008;**26**:421–9.
10. **Distler JH**, Allanore Y, Avouac J, *et al.* EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells. *Ann Rheum Dis* 2009;**68**:163–8.
11. **Masi AT**, Rodnan GP, Medsger TA Jr, *et al.* Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980;**23**:581–90.
12. **Prater DN**, Case J, Ingram DA, *et al.* Working hypothesis to redefine endothelial progenitor cells. *Leukemia* 2007;**21**:1141–9.
13. **Steinmetz M**, Nickenig G, Werner N. Endothelial-regenerating cells: an expanding universe. *Hypertension* 2010;**55**:593–9.
14. **Richardson MR**, Yoder MC. Endothelial progenitor cells: quo vadis? *J Mol Cell Cardiol* 2011;**50**:266–72.
15. **Timmermans F**, Plum J, Yöder MC, *et al.* Endothelial progenitor cells: identity defined? *J Cell Mol Med* 2009;**13**:87–102.

Experimental Hematology

Experimental Hematology 2012;40:279–289

Critical role of CD4$^+$CD25$^+$ regulatory T cells in preventing murine autoantibody-mediated thrombocytopenia

Tetsuya Nishimoto[a], Takashi Satoh[a,b], Tsutomu Takeuchi[a], Yasuo Ikeda[c], and Masataka Kuwana[a]

[a]*Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan;* [b]*Division of Hematology, Department of Medical Laboratory Sciences, Kitasato University School of Allied Health Sciences, Tokyo, Japan;* [c]*Faculty of Science and Engineering, Waseda University, Tokyo, Japan*

(Received 24 September 2011; revised 5 December 2011; accepted 3 January 2012)

Autoimmune response suppression by regulatory T cells (Tregs) helps to maintain peripheral immune tolerance, and defects in this mechanism are thought to play a role in the pathogenesis of various autoimmune diseases. In patients with immune thrombocytopenia, naturally occurring CD4$^+$CD25$^+$ Tregs are both functionally impaired and reduced in number. This study was undertaken to investigate Tregs' role in preventing immune thrombocytopenia in mice. Treg-deficient mice were prepared by inoculation of Treg-depleted CD4$^+$CD25$^-$ T cells isolated from BALB/c mice into syngeneic nude mice intravenously. Platelet count, proportion of reticulated platelets, platelet-associated IgG, platelet-associated anti-platelet antibodies, and IgG anti-platelet antibody production in splenocyte cultures were examined by flow cytometry. Of 69 Treg-deficient mice, 25 (36%) spontaneously developed thrombocytopenia that lasted at least 5 weeks. The platelet-associated IgG level and proportion of reticulated platelets were elevated in the thrombocytopenic mice. Platelet eluates and splenocyte culture supernatants prepared from thrombocytopenic mice, but not from nonthrombocytopenic mice, contained IgG antibodies capable of binding to intact platelets. Simultaneous transfer of Tregs completely prevented the onset of thrombocytopenia, but Treg transfer after the onset of thrombocytopenia had no apparent effect. Treatment with IgG anti-cytotoxic T lymphocyte−associated antigen 4 antibody canceled this Treg-governed suppressive effect. In summary, these results indicate that Tregs play a critical role in preventing murine autoantibody-mediated thrombocytopenia by engaging cytotoxic T lymphocyte−associated antigen 4. **© 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

In immune thrombocytopenia (ITP), circulating platelets are opsonized by IgG anti-platelet autoantibodies and subsequently destroyed in the reticuloendothelial system [1]. The major autoimmune targets are platelet membrane glycoproteins (GPs), such as GPIIb/IIIa and GPIb/IX [2]. Although the pathogenesis of ITP is still uncertain, a series of studies have shown that autoreactive CD4$^+$ T cells play a primary role [3–7]. In ITP patients, autoreactive CD4$^+$ T cells that recognize GPIIb/IIIa have been shown to exert helper activity promoting the production of IgG anti-GPIIb/IIIa antibodies, which can bind normal platelets in vitro [8]. We have proposed a "pathogenic loop" model for the ongoing IgG anti-platelet autoantibody response in ITP patients [9]: macrophages in the reticuloendothelial system capture opsonized platelets via the Fcγ receptor, and present antigenic platelet-derived peptides to T cells. This activates autoreactive CD4$^+$ T cells that exert helper activity to stimulate the B-cell production of IgG anti-platelet antibodies, which in turn bind to circulating platelets.

Naturally occurring regulatory T cells (Tregs), defined by the expression of CD4, CD25, and the transcription factor forkhead box P3 (Foxp3), play a leading role in protecting an individual from autoimmunity, thus maintaining immune tolerance and homeostasis [10]. Tregs can suppress effector T-cell responses through a series of mechanisms, which include engaging cytotoxic T lymphocyte−associated antigen 4 (CTLA-4) in direct cell−cell contact. Mice and humans genetically lacking functional Foxp3 are characterized by severe inflammation and autoimmunity, indicating the importance of Tregs in preventing autoimmune

Offprint requests to: Masataka Kuwana, M.D., Ph.D., Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; E-mail: kuwanam@z5.keio.jp

0301-472X/$ - see front matter. Copyright © 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.
doi: 10.1016/j.exphem.2012.01.001

diseases [11]. These findings raised the possibility that impaired Treg-mediated immune regulation is involved in the development of human autoimmune diseases. In fact, inadequate numbers of Tregs and defects in Treg function have been reported in many human autoimmune diseases, including type 1 diabetes, multiple sclerosis, and systemic lupus erythematosus [12]. In this regard, several independent studies have found significantly decreased Treg percentages in adults and children with ITP, compared with patients in remission or healthy individuals [13–16]. Tregs from ITP patients are defective in their ability to inhibit conventional CD4$^+$ T-cell proliferation [17]. Finally, a recent study by Bao et al. showed that Treg function was recovered in ITP patients who responded to thrombopoietin receptor agonists [18]. These studies together suggest that Tregs are critical for suppressing ITP pathogenic processes, but it remains unclear how incompetent Tregs allow autoimmune effector mechanisms to elicit ITP. In this study, we used Treg-deficient mice to investigate Tregs' in vivo role in ITP prevention.

Materials and methods

Mice

Female BALB/c mice, female BALB/c nu/nu mice, and male (NZW × BXSB) F1 mice were purchased from Sankyo Lab Service (Tokyo, Japan) and were used at 6 to 10 weeks of age. Experimental protocols were approved by the Keio University Ethics Committee for Animal Experiments.

Flow cytometric analysis

Cells were stained with fluorescein isothiocyanate or PC5-conjugated anti-CD4 monoclonal antibody (mAb) (clone GK1.5; BD Biosciences, San Diego, CA, USA) with or without fluorescein isothiocyanate–conjugated anti-CD25 (clone 7D4; BD Biosciences) or anti-Foxp3 mAb (clone FJK-16s; eBioscience, San Diego, CA, USA). For Foxp3 staining, the cells were fixed and permeabilized before incubation with the antibody according to manufacturer's instructions. Cells incubated with a species- and isotype-matched mAb to an irrelevant antigen were used for negative controls. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

Cell preparation

Splenocytes were isolated using Lymphosepar II (Immuno-Biological Laboratories, Takasaki, Japan) density-gradient centrifugation. Treg-depleted CD4$^+$ T cells and Tregs were prepared from splenocytes as CD4$^+$CD25$^-$ cells and CD4$^+$CD25$^+$ cells, respectively, by a magnetic-activated cell sorting system using a CD4$^+$CD25$^+$ Regulatory T Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4$^+$ cells were negatively selected using a cocktail of lineage-specific mAbs to CD8, CD11b, CD45R, CD49b, and Ter-119, followed by negative or positive selection of CD25$^+$ cells. The purity of Treg-depleted CD4$^+$ T cells and Tregs, as determined by flow cytometry, was >98% and >95%, respectively. In some experiments, CD4$^+$ T cells or CD19$^+$ B cells were depleted from splenocytes using the magnetic-activated cell sorting system. CD4$^+$ or CD19$^+$ cell contamination in the depleted fractions was <3%, as determined by flow cytometry.

Establishment of Treg-deficient mice

Treg-deficient mice were established as described previously [19] with some modifications. Briefly, Treg-depleted CD4$^+$ T cells (0.5, 2, or 5 × 10^7) were transferred intravenously into BALB/c nude mice. Recipient mice were followed for at least 8 weeks, and heparinized peripheral blood was collected from the retro-orbital plexus every week.

Protocols for the adoptive transfer of Tregs to Treg-deficient mice differed for prevention and treatment. For prevention, Tregs (2.5 × 10^6) were transferred simultaneously at the generation of Treg-deficient mice. For treatment, Tregs (2.5 × 10^6) were transferred 6 weeks after the generation of Treg-deficient mice after onset of thrombocytopenia was confirmed. Mock-treated Treg-deficient mice were also prepared as a control. In some instances, hamster anti–CTLA-4 mAb (IgG isotype, clone UC10-4F10-11; Bio X Cell, West Lebanon, NH, USA) or control hamster IgG (500 μg) was given twice a week for 2 weeks after adoptive Treg transfer for prevention [20].

Morphology and counts of blood and bone marrow cells

Morphology of peripheral blood cells was assessed by preparation of peripheral blood smear followed by Hemacolor staining (Merck, Whitehouse Station, NJ, USA). Leukocytes and erythrocytes were counted by microscopic observation using a Bürker–Türk counting chamber. To measure platelet count, whole blood was stained with fluorescein isothiocyanate–labeled anti-CD41 mAb (clone MWReg30; BD Biosciences) and examined by flow cytometry. Platelets were detected as CD41$^+$ cells within an adequate scatter gating area, and were counted using FlowCount microbeads (Beckman Coulter, Fullerton, CA, USA). Based on serial platelet counts in (NZW × BXSB) F1 mice, which spontaneously develop immune thrombocytopenia [21]. Thrombocytopenia was defined as a platelet count ≤0.33 × 10^6/μL. The morphology and number of megakaryocytes in bone marrow were evaluated according to the published method [22].

Reticulated platelets

Reticulated platelets were detected by staining whole blood with Retic-COUNT reagent (BD Biosciences). Platelets incubated with phosphate-buffered saline were prepared as mock-treated control. The proportion of reticulated platelets was measured by flow cytometry as described previously [23].

Platelet-associated IgG

Whole blood was incubated with AlexaFluor 488–labeled anti-mouse or anti-human IgG polyclonal antibodies (Invitrogen, Eugene, OR, USA) and subjected to flow cytometry. After adequate gating, the amount of IgG bound to platelets was quantified as a mean fluorescence index (MFI). Platelet-associated IgG (PAIgG) was then calculated as an MFI ratio of platelets treated with anti-mouse IgG to those treated with anti-human IgG.

PAIgG anti-platelet antibodies

Platelet eluates were prepared by incubating platelets with diethyl ether as described previously [24]. Circulating platelets derived from BALB/c mice were incubated with or without platelet eluates, and subsequently with AlexaFluor 488–labeled

anti-mouse IgG antibodies. The amount of antibodies bound to platelets was measured by flow cytometry as an MFI ratio of platelets treated with platelet eluates and anti-mouse IgG to those treated with anti-mouse IgG alone.

IgG anti-platelet antibody production in splenocyte cultures
Splenocytes (5×10^6) were cultured in 12-well plastic plates in RPMI 1640 plus 10% fetal bovine serum (Hyclone, Logan, UT, USA) for 4 days, and culture supernatants were collected by centrifugation. In some experiments, $CD4^+$ cell-depleted, $CD19^+$ cell-depleted, or mock-treated splenocytes were also used in the culture. Platelets derived from BALB/c mice were incubated with or without culture supernatants, and subsequently with AlexaFluor 488–labeled anti-mouse IgG antibodies. The amount of IgG anti-platelet antibodies was measured by flow cytometry as an MFI ratio of platelets treated with culture supernatants and anti-mouse IgG to those treated with anti-mouse IgG alone.

Detection of anti-GP antibodies in plasma
Antibodies to GPIIb and GPIIIa were detected in plasma by immunoblots using platelets as an antigen according to the published method [25]. Whole platelets were separated by electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide gels under reducing conditions, and then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with plasma diluted at 1:100, followed by incubation with horseradish peroxidase–conjugated rat anti-mouse IgG polyclonal antibodies (Thermo Fisher Scientific, Waltham, MA, USA). Rabbit polyclonal antibodies to GPIIb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GPIIIa (Merck Millipore, Darmstadt, Germany) in combination with horseradish peroxidase–conjugated anti-rabbit IgG antibodies were used as controls for the molecular sizes of corresponding GPs. The antibodies bound to the membranes were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Waltham, MA, USA).

Detection of anti-parietal cell antibodies
Anti-parietal cell antibodies were detected as described elsewhere [19], with some modifications. Briefly, frozen stomach sections (8-μm thick) prepared from BALB/c mice were fixed in cold acetone and incubated with plasma samples from Treg-deficient mice, serially diluted at 1:40, 1:160, and 1:640. Pooled nude mouse plasma was used as a negative control. Sections were then treated with AlexaFluor 488–labeled anti-mouse IgG antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (Invitrogen). Slides were examined with a fluorescence microscope (Olympus, Tokyo, Japan). The highest plasma dilution that gave a significant signal was regarded as the antibody titer. Anti-parietal cell antibodies were considered positive at a titer ≥1:40. Autoimmune gastritis was defined as having anti-parietal cell antibodies and histology of the gastric mucosa described previously [26].

Statistical analyses
Continuous values are shown as the mean ± standard deviation. Comparisons between two groups were tested for statistical significance using the nonparametric Mann–Whitney *U*-test or the Fisher's exact test as appropriate. A p value <0.05 was considered significant.

Results

Spontaneous thrombocytopenia in Treg-deficient mice
Treg-deficient mice were generated by transferring Treg-depleted $CD4^+$ T cells into syngeneic nude mice. The contamination of $CD25^+$ or $Foxp3^+$ cells in the conventional T-cell fraction was $<0.3\%$ (Fig. 1A). Four weeks after the transfer, engraftment of the transferred T cells was confirmed by detecting $CD4^+$ T cells in the circulation of recipient mice (Fig. 1B). Unexpectedly, we found that some mice spontaneously developed purpura in association with a low platelet count 3 or more weeks after the transfer (Fig. 1C). There was no morphologic abnormality in the peripheral blood smear or a decreased count of leukocytes or erythrocytes. Of 69 Treg-deficient mice, 25 (36%) developed thrombocytopenia at 3 weeks, with a duration of at least 5 weeks (Fig. 1D). Long-term observation of four thrombocytopenic mice revealed that the thrombocytopenia persisted >20 weeks, but none of them died of bleeding. In contrast, all the mice that were not thrombocytopenic at 4 weeks after the transfer maintained a normal platelet count during an additional 8 weeks of follow-up. Serial measurements revealed that the reticulated platelets and PAIgG increased in the thrombocytopenic mice, but not in the nonthrombocytopenic mice. Elevated levels of reticulated platelets and PAIgG were apparent 2 weeks after the transfer, before the onset of thrombocytopenia (Fig. 2). The morphology and number of megakaryocytes in the bone marrow at 4 weeks after the transfer were comparable between Treg-deficient mice with and without thrombocytopenia.

IgG anti-platelet autoantibodies in thrombocytopenic mice
To evaluate whether the thrombocytopenia in Treg-deficient mice was associated with IgG anti-platelet autoantibodies, flow cytometry was used to examine platelet eluates prepared from Treg-deficient mice with or without thrombocytopenia for their ability to bind to intact platelet surfaces. Platelet eluates obtained from a representative thrombocytopenic mouse contained IgG capable of binding platelets, whereas those from a nonthrombocytopenic mouse did not (Fig. 3A). The level of platelet-associated IgG anti-platelet antibodies was significantly increased in the platelet eluates obtained from 14 thrombocytopenic mice compared with those from eight nonthrombocytopenic mice (2.4 ± 2.4 vs 1.0 ± 0.1; $p < 0.01$) (Fig. 3B), indicating a correlation between IgG anti-platelet autoantibody production and thrombocytopenia.

Antibodies to platelet GPs in thrombocytopenic mice
To examine the autoantibody response to platelet GPs in thrombocytopenic mice, plasma samples from 10 thrombocytopenic mice were subjected to immunoblots using platelets as antigen. As shown in Figure 4, three and one reacted

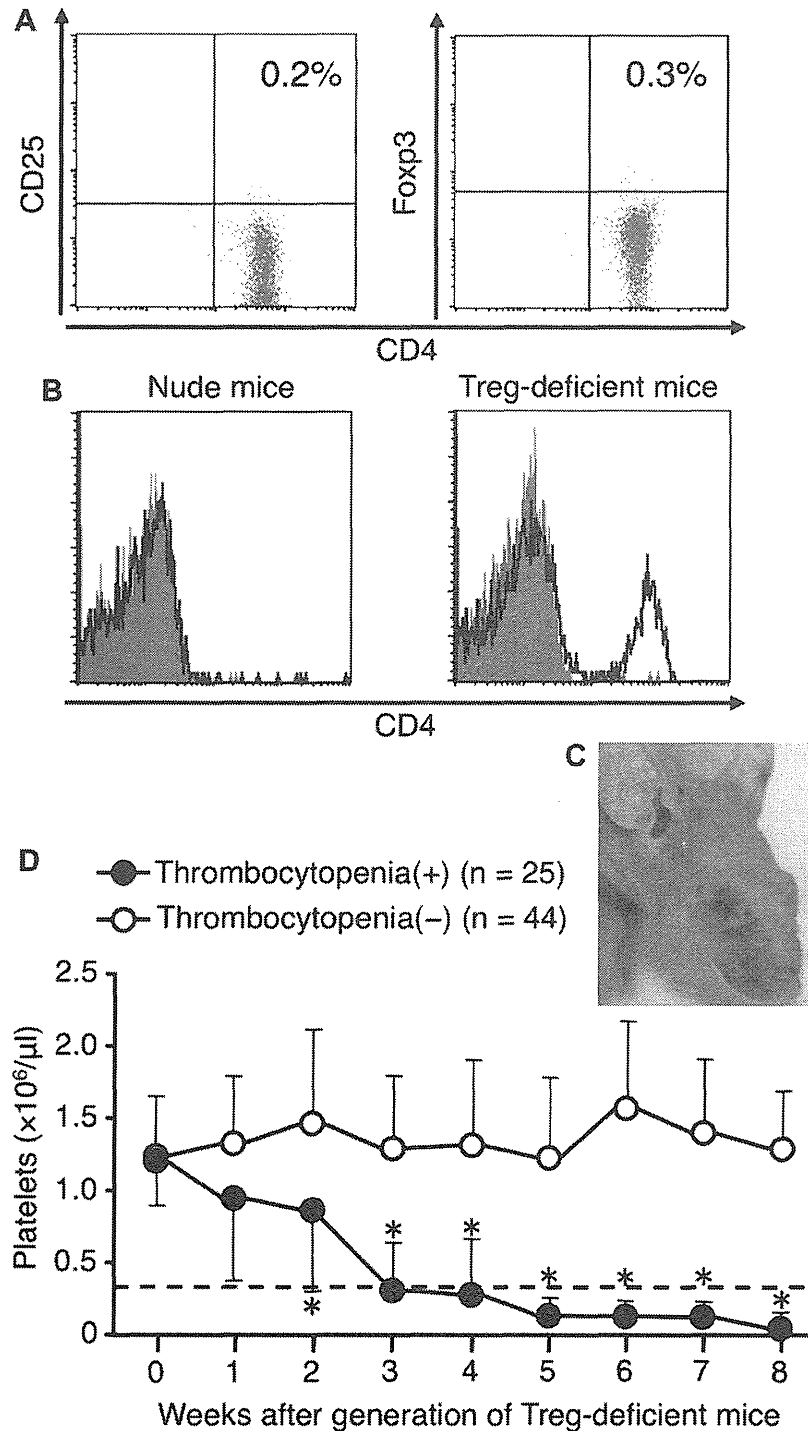

Figure 1. Thrombocytopenia in Treg-deficient mice. (**A**) Treg-depleted CD4$^+$ T cells isolated from splenocytes using a magnetic-activated cell sorting system were stained with an anti-CD4 mAb and either an anti-CD25 or anti-Foxp3 mAb, and examined by flow cytometry. Representative results are shown. The frequency of CD4$^+$CD25$^+$ or CD4$^+$Foxp3$^+$ cells in the Treg-depleted CD4$^+$ T-cell fraction is shown as a percentage in the upper right of each panel. (**B**) Peripheral blood obtained from nude or Treg-deficient mice 4 weeks after the induction of Treg deficiency was stained with the anti-CD4 mAb and subjected to flow cytometry. Representative results are shown. (**C**) A typical image of a Treg-deficient mouse with prominent subcutaneous hemorrhage. (**D**) Serial platelet counts in Treg-deficient mice divided according to the presence or absence of thrombocytopenia 3 weeks after Treg deficiency was induced. A dashed line indicates the cutoff level, defined as a platelet count of $0.33 \times 10^6/\mu L$. Results are shown as the mean and standard deviation. *Statistically significant differences between thrombocytopenic and nonthrombocytopenic mice ($p < 0.01$ for all comparisons).

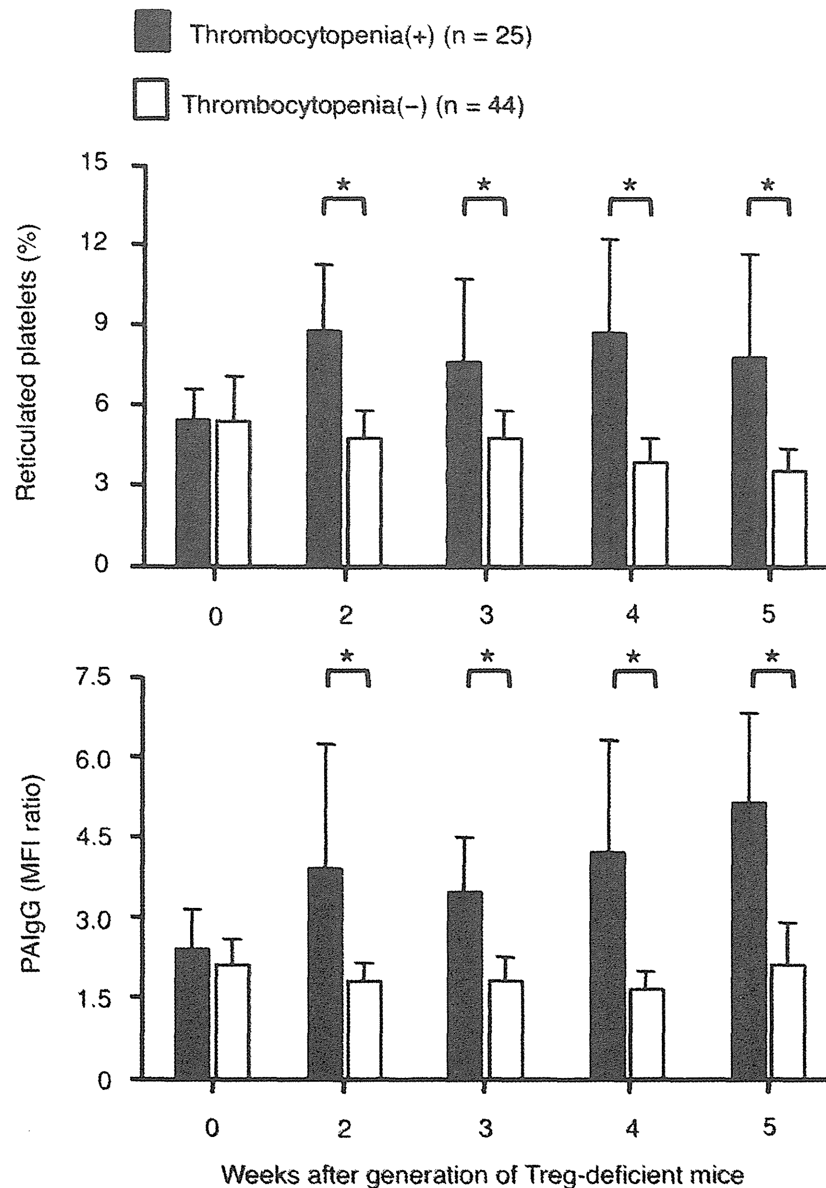

Figure 2. Serial measurement of reticulated platelets and PAIgG in Treg-deficient mice. The percentage of reticulated platelets (top) and PAIgG level (bottom) were measured 0, 2, 3, 4, and 5 weeks after the generation of Treg-deficient mice. Closed and open bars show the mean and standard deviation of 25 thrombocytopenic mice and 44 nonthrombocytopenic mice, respectively. *Statistically significant differences between thrombocytopenic and nonthrombocytopenic mice ($p < 0.01$ for all comparisons).

with the proteins with molecular sizes corresponding to GPIIb and GPIIIa, respectively. In contrast, recognition of those platelets GPs was detected in none of plasma samples from 12 nonthrombocytopenic mice.

Ex vivo splenocyte production of IgG anti-platelet autoantibodies

Splenocytes obtained from thrombocytopenic and nonthrombocytopenic mice were cultured without antigenic or mitogenic stimulation, and supernatants were examined for the ex vivo production of IgG anti-platelet antibodies. Although spontaneous IgG anti-platelet antibody production was detected in the majority of splenocytes cultured from thrombocytopenic mice, it was not detected in any culture from nonthrombocytopenic mice. IgG anti-platelet antibodies were produced at a significantly higher level in splenocytes cultures from thrombocytopenic mice than in those from nonthrombocytopenic mice (6.3 ± 8.2 vs 1.0 ± 0.1; $p < 0.01$) (Fig. 5A). Depleting the $CD19^+$ B cells or $CD4^+$ T cells from the splenocytes partially

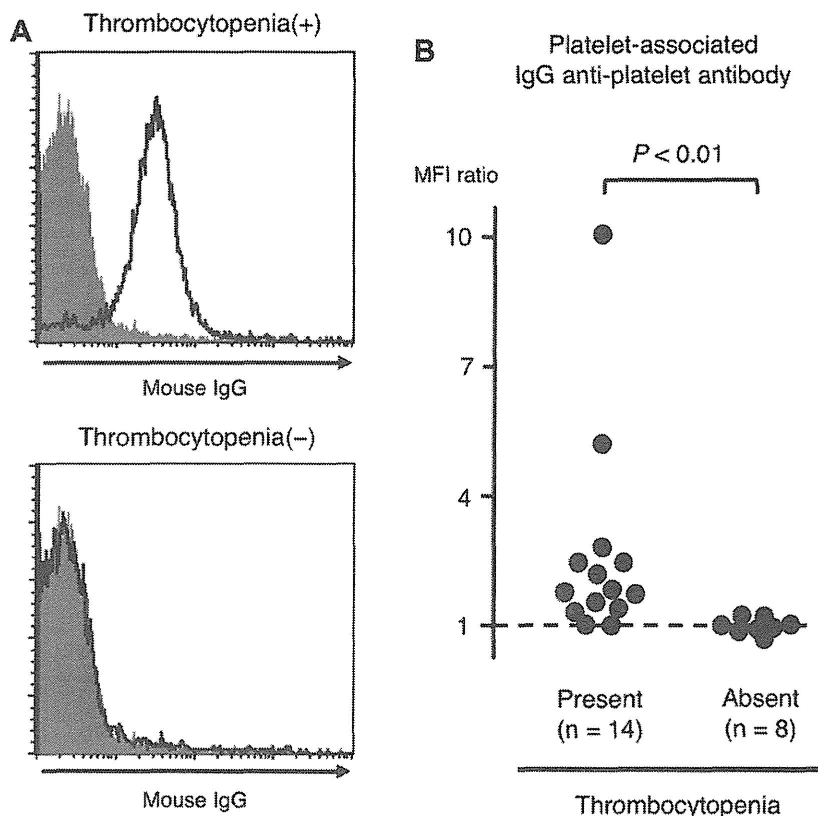

Figure 3. Platelet-associated IgG anti-platelet antibodies in Treg-deficient mice. BALB/c platelets were incubated with or without platelet eluates, and then with anti-mouse IgG antibodies. IgG anti-platelet antibodies were measured by flow cytometry as an MFI ratio of platelets treated with platelet eluates and anti-mouse IgG to those treated with anti-mouse IgG alone. (**A**) Representative flow cytometric findings in thrombocytopenic and nonthrombocytopenic mice. Nonshaded histograms represent staining with platelet eluates and anti-mouse IgG, while shaded histograms represent staining with anti-mouse IgG alone. (**B**) Proportion of platelet-associated IgG anti-platelet antibodies in 14 thrombocytopenic mice and 8 nonthrombocytopenic mice.

inhibited the anti-platelet antibody production (Fig. 5B). These findings suggest that, in splenocyte culture, IgG anti-platelet autoantibodies are produced mainly from B cells and partially from $CD19^-$ plasma cells or plasmablasts. In addition, this process depends, in part, on an interaction between activated $CD4^+$ T cells and B cells.

Relationship between the incidence of thrombocytopenia and the number of Treg-depleted $CD4^+$ T cells transferred

Autoantibody-mediated thrombocytopenia was observed in only a subset of the Treg-deficient mice. To examine whether the incidence of thrombocytopenia depends on the number of Treg-depleted $CD4^+$ T cells transferred, Treg-deficient mice were generated by transferring different numbers of Treg-depleted $CD4^+$ T cells (0.5, 2, or 5×10^7). Three weeks after the transfer, thrombocytopenia was observed in only 2 (8%) of the 24 mice given the lowest number of T cells (0.5×10^7); this incidence was significantly less than that in mice given 2×10^7 T cells (36%; $p < 0.01$). However, none of the mice given the highest number of Treg-depleted $CD4^+$ T cells (5×10^7) developed thrombocytopenia, indicating that a correlation between the incidence of thrombocytopenia and the number of Treg-depleted $CD4^+$ T cells transferred is not simple.

Relationship between autoantibody-mediated thrombocytopenia and gastritis

It has been reported that Treg-deficient mice develop several distinct organ-specific autoimmune diseases [19]. Of these, autoimmune gastritis mediated by IgG anti-parietal cell antibodies is the most common. To examine the potential relationship between the onset of thrombocytopenia and gastritis, IgG anti-parietal cell antibody titers were measured in the plasma derived from Treg-deficient mice 8 weeks after the transfer. Immunofluorescent images showed IgG antibodies binding to parietal cells in the stomach sections in the majority of mice (Fig. 6A). Frequencies of autoimmune gastritis in thrombocytopenic and nonthrombocytopenic mice were comparable (60% and 75%, respectively). In addition, no difference was found between the IgG anti-parietal cell antibody titers in the thrombocytopenic and nonthrombocytopenic mice (Fig. 6B), indicating that autoimmune thrombocytopenia and gastritis occurred independently of each other.

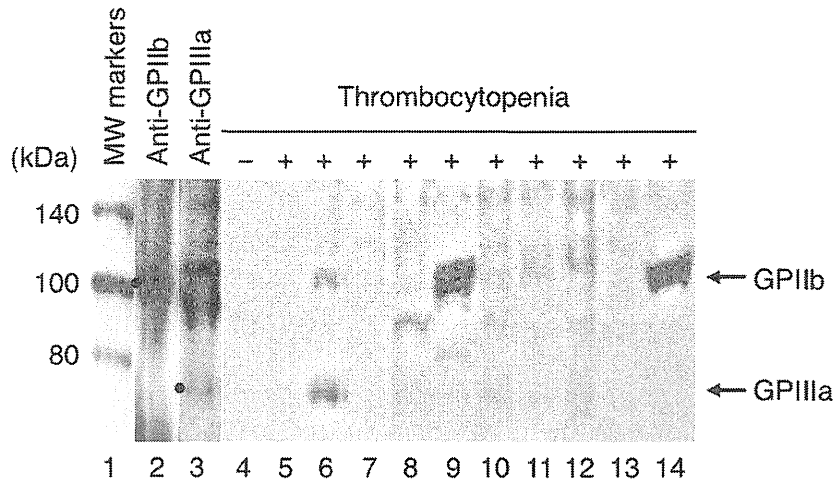

Figure 4. Binding of plasma autoantibodies to platelet GPIIb and GPIIIa in immunoblots. Whole platelets were separated under reducing conditions, transferred to a nitrocellulose membrane, and probed with plasma samples from nonthrombocytopenic mouse (lane 4) and thrombocytopenic mice (lanes 5–14). The following antibodies were used as controls: anti-GPIIb (lane 2) and anti-GPIIIa (lane 3). The molecular weights corresponding to GPIIb and GPIIIa are shown as dots. MW = molecular weight markers.

Adoptive Treg transfer to prevent autoantibody-mediated thrombocytopenia

To confirm that the Treg deficiency was responsible for the onset of autoantibody-mediated thrombocytopenia, Tregs were adoptively transferred at the same time that the Treg-deficient mice were generated. As expected, simultaneous Treg transfer completely prevented the onset of thrombocytopenia in all 17 recipient mice, whereas 6 of 19 mice treated with mock transplantation developed thrombocytopenia (0% vs 32%; $p < 0.05$). In contrast,

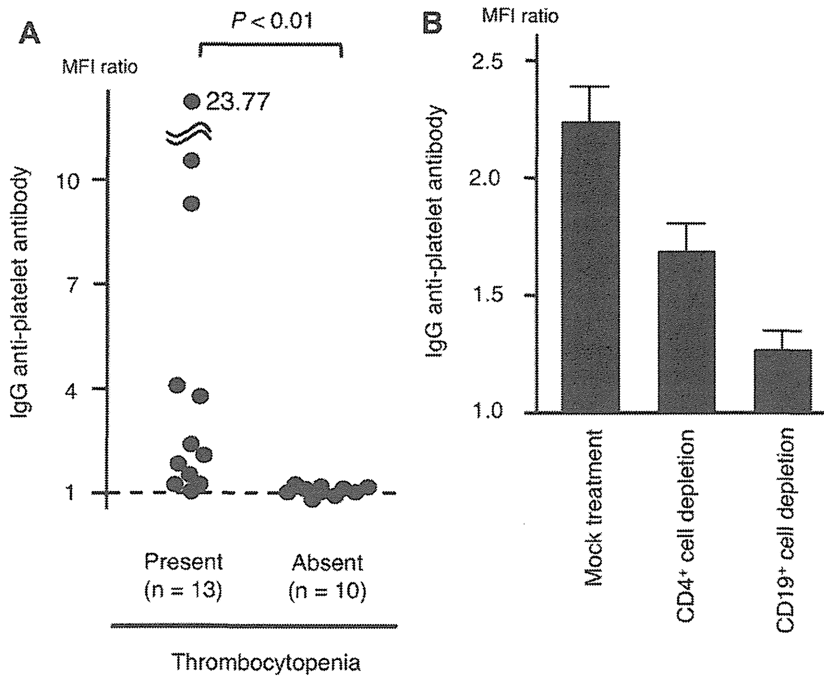

Figure 5. Ex vivo production of IgG anti-platelet antibodies in splenocytes from Treg-deficient mice. Splenocytes were cultured for 4 days and then centrifuged to collect the culture supernatants. BALB/c platelets were incubated with or without these supernatants, and subsequently with anti-mouse IgG antibodies. IgG anti-platelet antibodies were measured by flow cytometry as an MFI ratio of platelets treated with culture supernatants and anti-mouse IgG to those treated with anti-mouse IgG alone. (**A**) IgG anti-platelet antibodies produced in splenocyte cultures of 13 thrombocytopenic mice and 10 nonthrombocytopenic mice. (**B**) IgG anti-platelet antibodies produced in cultures of mock-treated splenocytes, $CD4^+$ cell-depleted splenocytes, and $CD19^+$ cell-depleted splenocytes. The mean and standard deviation of four independent experiments are shown.

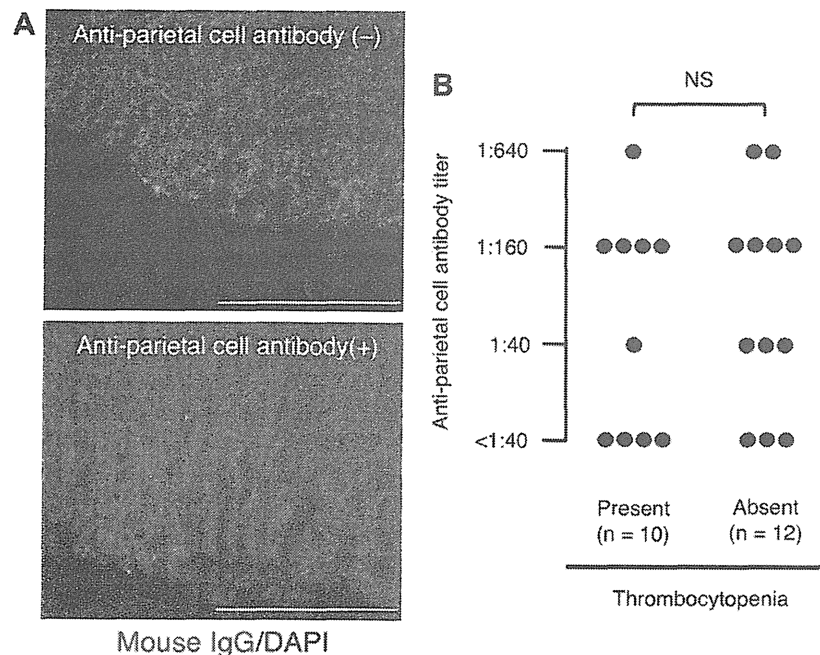

Figure 6. Anti-parietal cell antibodies in Treg-deficient mice according to the presence or absence of thrombocytopenia. Frozen stomach sections prepared from BALB/c mice were fixed in cold acetone and incubated with plasma samples serially diluted at 1:40, 1:160, and 1:640. Sections were then treated with AlexaFluor 488-labeled anti-mouse IgG antibodies (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (blue). The slides were examined with a fluorescence microscope. (**A**) Representative immunofluorescent images of sera negative (upper) and positive (lower) for IgG anti-parietal cell antibodies. Scale bars: 200 μm. (**B**) Anti-parietal cell antibody titers in 10 thrombocytopenic mice and 12 nonthrombocytopenic mice. The highest plasma dilution that gave a positive signal was regarded as the antibody titer. NS = not significant.

adoptive Treg transfer after the onset of thrombocytopenia in five mice did not increase the platelet count or suppress the anti-platelet autoantibody response.

To further examine the mechanisms responsible for Tregs' suppression of the autoantibody responses to platelets, anti−CTLA-4 or control antibodies were given to Treg-deficient mice with or without adoptive Treg transfer (Table 1). Treatment with an anti−CTLA-4 mAb cancelled the Treg transfer's protective effect against thrombocytopenia, while treatment with control antibodies had no effect. It has been shown that antibody-mediated CTLA-4 blockade augments effector T-cell responses [27], but in the absence of the Treg transfer, treating Treg-deficient mice with the anti−CTLA-4 mAb did not increase the incidence of thrombocytopenia. These findings indicate that Tregs prevent the development of autoantibody-mediated thrombocytopenia through CTLA-4.

Table 1. Incidence of thrombocytopenia in Treg-deficient mice that received or did not receive the adoptive transfer of Tregs for prevention, according to treatment with anti−CTLA-4 mAb or control IgG

Adoptive transfer of Tregs	Antibody treatment	Mice examined (n)	Mice with thrombocytopenia, n (%)
+	Anti−CTLA-4	16	3 (19)*
+	Control IgG	20	0 (0)
−	Anti-CTLA-4	10	2 (20)
−	Control IgG	11	2 (18)

*$p < 0.05$ compared with mice given both Tregs and control IgG.

Discussion

In this study, we demonstrated that depleting $CD4^+CD25^+$ Tregs in mice resulted in the spontaneous onset of chronic thrombocytopenia mediated by IgG anti-platelet autoantibodies. An increased proportion of reticulated platelets, along with elevated PAIgG and platelet-associated IgG anti-platelet antibodies, were detected in the thrombocytopenic mice; this reflects de novo IgG anti-platelet autoantibody production and enhanced platelet turnover, and is analogous to laboratory results observed in patients with ITP [28]. In addition, IgG autoantibodies to platelet GPIIb and GPIIIa were specifically produced in some thrombocytopenic mice, indicating that GPIIb/IIIa is one of the autoimmune targets, although the antibodies to denatured GPs detected by immunoblots might be unrelated to pathogenic antibodies that bind to native platelets. This simple model generated by transfer of Treg-depleted $CD4^+$ T cells into nude mice is a new mouse model that mimics the pathophysiology of human ITP.

It has been shown that autoantibody-mediated and T cell-mediated autoimmune responses to bone marrow megakaryocytes are involved in the pathogenic processes of human ITP [29,30]. On the other hand, in a recently established isoimmune thrombocytopenic mouse model, which was generated by transferring splenocytes from GPIIIa knockout mice into severe combined immunodeficient mouse possessing GPIIIa$^+$ platelets, cytotoxic T-cell–mediated destruction of megakaryocytes and resultant ineffective thrombopoiesis play an important role in induction of thrombocytopenia [22]. However, lack of morphologic and quantitative alternations in bone marrow megakaryocytes and increased proportion of reticulated platelets in our model suggest the primary role of the antibody-mediated platelet destruction in thrombocytopenia at least in the short-term.

Our results clearly showed that transfer of Treg-depleted CD4$^+$ T cells into nude mice is sufficient to induce an autoantibody response to platelets, leading to the onset of autoantibody-mediated thrombocytopenia. Adoptive Treg transfer at the time of the generation of Treg-deficient mice completely prevented the onset of thrombocytopenia, confirming the Tregs' critical role in preventing autoantibody-mediated thrombocytopenia. Tregs act mainly through antigen-presenting cells (APCs), such as dendritic cells and macrophages, to limit the priming and activation of autoreactive T cells, thus preventing their differentiation and acquisition of effector functions [31]. Autoreactive CD4$^+$ T cells responsive to platelet antigens such as GPIIb/IIIa are present in the circulation of both ITP patients and healthy individuals, and are thus a component of the normal T-cell repertoire [8,32]. In our mouse model, transferred Treg-depleted CD4$^+$ T cells (most likely including a small fraction of autoreactive T cells) would expand rapidly through homeostatic proliferation, a process that occurs in lymphopenic hosts to replenish the T-cell pool [33]. In this process, Tregs control the reconstitution of the T-cell repertoire by inhibiting the excessive expansion of autoreactive T cells [34]. However, in the absence of Tregs, harmful autoimmune responses could be driven by the expansion and activation of autoreactive T cells residing in the Treg-depleted CD4$^+$ T-cell fraction. In fact, Treg-deficient mice are known to develop a variety of organ-specific autoimmune diseases, including thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, and oophoritis [19]. Therefore, the absence of Tregs during homeostatic proliferation is responsible for the induction of various organ-specific autoimmune diseases. Moreover, autoantibody-mediated thrombocytopenia is one of the autoimmune conditions observed in Treg-deficient mice, and occurs secondary to immune dysregulation prone to developing harmful immune responses to a series of autoantigens. Taken together, Tregs play a key role in maintaining immune tolerance to platelets by suppressing the activation of autoreactive CD4$^+$ T cells within the normal repertoire. Thus, it is likely that incompetent Treg function greatly increases susceptibility to ITP.

Tregs maintain immune tolerance by actively suppressing effector T-cell responses, either directly or by acting on APCs through various mechanisms [35]. Tregs can deliver a negative signal to target cells by direct cell–cell contact through CTLA-4 or by generating suppressive soluble factors such as transforming growth factor–β and interleukin-10. Tregs also suppress effector T-cell function by competing for interleukin-2 and APC interactions. Of these possible mechanisms, the Tregs' suppressive function on autoantibody-mediated thrombocytopenia was mediated primarily through CTLA-4, which is constitutively expressed on Tregs. CTLA-4 competes with CD28 to bind to CD80/CD86 on APCs, and inhibits an interaction between CD28 and CD80/CD86 that is critical for primary T-cell responses [36]. The importance of CTLA-4 in Treg function has been shown in mice genetically deficient in CTLA-4 selectively on Tregs, which spontaneously develop fatal T-cell-mediated autoimmune diseases [37].

Interestingly, more than half of the Treg-deficient mice generated did not develop thrombocytopenia or an antiplatelet autoantibody response, even in the absence of Tregs. What determines whether Treg-deficient mice will develop autoantibody-mediated thrombocytopenia or not? In this regard, patterns of autoimmune diseases have been shown to differ among Treg-deficient mice [19]. In addition, we found that the occurrence of thrombocytopenia and gastritis in Treg-deficient mice were unrelated. This is probably because individual autoimmune conditions were mediated through distinct autoreactive T-cell populations. Our experiments varying the quantity of Treg-depleted CD4$^+$ T cells used to generate the Treg-deficient mice suggested that the number of specific autoreactive CD4$^+$ T cells included in the transferred T cells may be one of the factors determining if the recipient mice will develop autoimmune disease or not. Autoantibody-mediated thrombocytopenia might occur if the T-cell population reconstituted through homeostatic proliferation contains sufficient platelet antigen-reactive CD4$^+$ T cells to drive the autoantibody response. However, thrombocytopenia did not occur in any of the mice given the largest number of Treg-depleted CD4$^+$ T cells; this was probably due to a low degree of homeostatic proliferation, which was insufficient to fully activate the pathogenic autoreactive T cells. Additional studies are necessary to understand the mechanisms that control the expansion of autoreactive CD4$^+$ T cells specific to platelet antigens during homeostatic proliferation.

Adoptive cell therapy using ex vivo–expanded autologous Tregs is proposed as a promising treatment strategy for patients with autoimmune diseases [38]. Theoretically, transferred Tregs are expected to halt ongoing pathogenic autoimmunity and restore immune tolerance. However, in our model, adoptive Treg transfer after the onset of

thrombocytopenia failed to increase the platelet count. This is consistent with a previous report showing that adoptive Treg transfer has to be performed within a few days after the transplantation of Treg-depleted $CD4^+$ T cells to prevent autoimmune gastritis in Treg-deficient mice [19]. Because the Tregs' suppressive function is rather nonspecific, a large number of Tregs would be required to suppress an ongoing pathogenic response. Taken together with the functional impairment found in Tregs derived from patients with ITP [17], autologous Treg infusion therapy appears impractical for treating ITP. On the other hand, Zhang et al. recently reported the ex vivo induction and expansion of human GPIIb/IIIa-reactive Tregs, highlighting the considerable potential of these antigen-specific Tregs for selective immunotherapy for ITP [39].

In summary, our results are the first to show a critical role of $CD4^+CD25^+Foxp3^+$ Tregs in preventing autoantibody-mediated thrombocytopenia in vivo. The Treg-deficient mouse model is useful for analyzing the pathophysiology of ITP, especially in the induction phase, and in evaluating novel therapeutic strategies for ITP.

Funding disclosure
This work was supported by a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (21790926), and by a research grant on intractable diseases from the Japanese Ministry of Health, Labor and Welfare.

Conflict of interest disclosure
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

1. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. N Engl J Med. 2002;346:995–1008.
2. McMillan R. Autoantibodies and autoantigens in chronic immune thrombocytopenic purpura. Semin Hematol. 2000;37:239–248.
3. Semple JW, Freedman J. Increased antiplatelet T helper lymphocyte reactivity in patients with autoimmune thrombocytopenia. Blood. 1991;78:2619–2625.
4. Kuwana M, Ikeda Y. The role of autoreactive T-cells in the pathogenesis of idiopathic thrombocytopenic purpura. Int J Hematol. 2005;81:106–112.
5. Ware RE, Howard TA. Phenotypic and clonal analysis of T lymphocytes in childhood immune thrombocytopenic purpura. Blood. 1993;82:2137–2142.
6. Semple JW, Milev Y, Cosgrave D, et al. Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity. Blood. 1996;87:4245–4254.
7. Panitsas FP, Theodoropoulou M, Kouraklis A, et al. Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response. Blood. 2004;103:2645–2647.
8. 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.
9. 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.
10. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133:775–787.
11. Ochs HD, Gambineri E, Torgerson TR. IPEX, FOXP3 and regulatory T-cells: a model for autoimmunity. Immunol Res.. 2007;38:112–121.
12. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+) FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol. 2010;10:849–859.
13. Ling Y, Cao X, Yu Z, Ruan C. Circulating dendritic cells subsets and CD4+Foxp3+ regulatory T cells in adult patients with chronic ITP before and after treatment with high-dose dexamethasome. Eur J Haematol. 2007;79:310–316.
14. Liu B, Zhao H, Poon MC, et al. Abnormality of CD4(+)CD25(+) regulatory T cells in idiopathic thrombocytopenic purpura. Eur J Haematol. 2007;78:139–143.
15. Sakakura M, Wada H, Tawara I, et al. Reduced CD4+CD25+ T cells in patients with idiopathic thrombocytopenic purpura. Thromb Res. 2007;120:187–193.
16. Aboul-Fotoh Lel M, Abdel Raheem MM, El-Deen MA, Osman AM. Role of CD4+CD25+ T cells in children with idiopathic thrombocytopenic purpura. J Pediatr Hematol Oncol. 2011;33:81–85.
17. Yu J, Heck S, Patel V, et al. Defective circulating CD25 regulatory T cells in patients with chronic immune thrombocytopenic purpura. Blood. 2008;112:1325–1328.
18. 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.
19. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155:1151–1164.
20. Lepenies B, Gaworski I, Tartz S, Langhorne J, Fleischer B, Jacobs T. CTLA-4 blockade differentially influences the outcome of non-lethal and lethal Plasmodium yoelii infections. Microbes Infect. 2007;9:687–694.
21. Oyaizu N, Yasumizu R, Miyama-Inaba M, et al. (NZW x BXSB)F1 mouse. A new animal model of idiopathic thrombocytopenic purpura. J Exp Med. 1988;167:2017–2022.
22. Chow L, Aslam R, Speck ER, et al. A murine model of severe immune thrombocytopenia is induced by antibody- and $CD8^+$ T cell-mediated responses that are differentially sensitive to therapy. Blood. 2010;115:1247–1253.
23. Sun L, Liu X, Qiu L, et al. Administration of plasmid DNA expressing human interleukin-6 significantly improves thrombocytopoiesis in irradiated mice. Ann Hematol. 2001;80:567–572.
24. Musaji A, Vanhoorelbeke K, Deckmyn H, Coutelier JP. New model of transient strain-dependent autoimmune thrombocytopenia in mice immunized with rat platelets. Exp Hematol. 2004;32:87–94.
25. Kokawa T, Nomura S, Yanabu M, Yasunaga K. Detection of platelet antigen for antiplatelet antibodies in idiopathic thrombocytopenic purpura by flow cytometry, antigen-capture ELISA, and immunoblotting: a comparative study. Eur J Haematol. 1993;50:74–80.
26. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med. 1996;184:387–396.
27. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996;271:1734–1736.

28. Kuwana M, Kurata Y, Fujimura K, et al. Preliminary laboratory based diagnostic criteria for immune thrombocytopenic purpura: evaluation by multi-center prospective study. J Thromb Haemost. 2006;4:1936–1943.
29. McMillan R, Wang L, Tomer A, et al. Suppression of *in vitro* megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. Blood. 2004;103:1364–1369.
30. Houwerzijl EJ, Blom NR, de Wolf JT, et al. Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura. Blood. 2004;103:500–506.
31. Tang Q, Bluestone JA. Regulatory T-cell physiology and application to treat autoimmunity. Immunol Rev. 2006;212:217–237.
32. Filion MC, Proulx C, Bradley AJ, et al. Presence in peripheral blood of healthy individuals of autoreactive T cells to a membrane antigen present on bone marrow-derived cells. Blood. 1996;88:2144–2150.
33. Williams KM, Hakim FT, Gress RE. T cell immune reconstitution following lymphodepletion. Semin Immunol. 2007;19:318–330.
34. Shen S, Ding Y, Tadokoro CE, et al. Control of homeostatic proliferation by regulatory T cells. J Clin Invest. 2005;115:3517–3526.
35. Jiang S, Tsang J, Tam P. Regulatory T cell immunotherapy for transplantation tolerance: step into clinic. Int Immunopharmacol. 2010;10: 1486–1490.
36. Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity. 1997;6:411–417.
37. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008;322:271–275.
38. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. Immunity. 2009;30: 656–665.
39. Zhang XL, Peng J, Sun JZ, et al. De novo induction of platelet-specific CD4(+)CD25(+) regulatory T cells from CD4(+)CD25(-) cells in patients with idiopathic thrombocytopenic purpura. Blood. 2009; 113:2568–2577.

TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

Tissue Antigens ISSN 0001-2815

Associations between six classical *HLA* loci and rheumatoid arthritis: a comprehensive analysis

S. Mitsunaga[1], Y. Suzuki[2], M. Kuwana[3], S. Sato[2], Y. Kaneko[3], Y. Homma[4], A. Narita[1], K. Kashiwase[5], Y. Okudaira[1], I. Inoue[1,6], J. K. Kulski[1,7] & H. Inoko[1]

1 Department of Molecular Life Sciences, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Isehara, Japan
2 Division of Rheumatology, Department of Internal Medicine, Tokai University School of Medicine, Isehara, Japan
3 Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan
4 Department of Clinical Health Science, Tokai University School of Medicine, Isehara, Japan
5 Department of Laboratory, Japanese Red Cross Tokyo Blood Center, Tokyo, Japan
6 Division of Human Genetics, Department of Integrated Genetics, National Institute of Genetics, Mishima, Japan
7 Centre for Forensic Science, The University of Western Australia, Nedlands, WA 6008, Australia

Key words
association study; bone erosion; *HLA-DPB1*; *HLA* haplotypes; rheumatoid arthritis

Correspondence
Hidetoshi Inoko, PhD
Department of Molecular Life Science
Division of Basic Medical Science and Molecular Medicine
Tokai University School of Medicine
143 Shimokasuya
Isehara
Kanagawa 259-1193
Japan
Tel: +81 463 93 1121 (ext. 2312)
Fax: +81 463 94 8884
e-mail: hinoko@is.icc.u-tokai.ac.jp

Received 14 September 2011; revised 7 March 2012; accepted 15 March 2012

doi: 10.1111/j.1399-0039.2012.01872.x

Abstract

Although the *HLA* region contributes to one-third of the genetic factors affecting rheumatoid arthritis (RA), there are few reports on the association of the disease with any of the *HLA* loci other than the *DRB1*. In this study we examined the association between RA and the alleles of the six classical *HLA* loci including *DRB1*. Six HLA loci (*HLA-A, -B, -C, -DRB1, -DQB1* and *-DPB1*) of 1659 Japanese subjects (622 cases; 488 anti-cyclic citrullinated peptides (CCP) antibody (Ab) positive (82.6%); 103 anti-CCP Ab negative (17.4%); 31 not known and 1037 controls) were genotyped. Disease types and positivity/negativity for CCP autoantibodies were used to stratify the cases. Statistical and genetic assessments were performed by Fisher's exact tests, odds ratio, trend tests and haplotype estimation. None of the *HLA* loci were significantly associated with CCP sero-negative cases after Bonferroni correction and we therefore limited further analyses to using only the anti CCP-positive RA cases and both anti-CCP positive and anti-CCP negative controls. Some alleles of the non-*DRB1 HLA* loci showed significant association with RA, which could be explained by linkage disequilibrium with *DRB1* alleles. However, *DPB1*02:01*, *DPB1*04:01* and *DPB1*09:01* conferred RA risk/protection independently from *DRB1*. *DPB1*02:01* was significantly associated with the highly erosive disease type. The odds ratio of the four HLA-loci haplotypes with *DRB1*04:05* and *DQB1*04:01*, which were the high-risk *HLA* alleles in Japanese, varied from 1.01 to 5.58. *C*07:04*, and *B*15:18* showed similar *P*-values and odds ratios to *DRB1*04:01*, which was located on the same haplotype. This haplotype analysis showed that the *DRB1* gene as well as five other *HLA* loci is required for a more comprehensive understanding of the genetic association between *HLA* and RA than analyzing *DRB1* alone.

Introduction

Rheumatoid arthritis (RA) is a multifactorial and systemic autoimmune disease that can lead to progressive joint destruction and disability. About 60% of the RA risk is genetic, and the disease concordance rate of RA is about 12%–15% in monozygotic twins, 3%–4% in dizygotic twins, and 2%–4% in non-twin siblings (1) The ratio of the risk of disease recurrence among siblings of affected individuals to disease incidence in the general population is 5–10 (2). Many genome-wide association studies have been conducted to identify genetic factors that contribute to RA (3, 4) and more than 30 non-HLA loci associated with RA have been reported (5). The non-HLA loci, which were reported to be RA-susceptible genes, so far explain only about 5% of genetic variance (5, 6). The remaining genetic variance is explained by rare variants (7) and genetic interactions (8). One-third of the genetic risk of RA is in the major histocompatibility complex

© 2012 John Wiley & Sons A/S

(MHC, human leukocyte antigens in human: HLA) region (9) with *HLA-DRB1* being the most susceptible gene. The major *DRB1* risk alleles are *DRB1*04:01* and *DRB1*04:04* in Caucasians and *DRB1*04:05* in East Asian populations (10). The shared epitope (SE), which is defined by the amino acid sequence at the positions 70–74 of the HLA-DR beta chain, is associated with susceptibility to RA (11, 12) and with the structural severity of the disease (13). These residues constitute a helical domain and form one side of the antigen binding site that is likely to affect antigen presentation (10).

The human MHC genomic region is divided into class I and class II MHC with the former consisting of three genes, *HLA-A, -B, -C,* and the latter of three antigen gene families, *-DR, -DQ, -DP.* The Class I antigens, HLA-A, -B, -C, consist of a heavy chain and β2-micro globulin with highly polymorphic regions on the heavy chain. The Class II antigens, HLA-DR, -DQ, -DP, consist of alpha and beta chains, with highly polymorphic regions on the beta chain encoded by the *HLA-DRB1, -DQB1, -DPB1* genes. The MHC Class III region, which is mapped between class I and class II regions, also contains some RA-susceptible genes (14–22) such as *AIF1* (14, 15) and *NFKBIL1* (16–18). The gene order of the six HLA loci from the telomere to the centromere is *HLA-A, -C, -B, -DRB1, -DQB1* and *-DPB1*, and their high and long-range linkage disequilibrium (LD) is one of the hallmarks of the MHC region (23). Although the major genetic risk factor for RA is the *HLA* region, only *HLA-DRB1* and the SE, which is defined by the five amino acids of the DR-β chain, have been studied in detail for their association with RA. However, the five *HLA* genes other than *DRB1* also need to be examined as they are highly polymorphic and may be associated with RA disease progression or protection.

Autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptides (CCP) antibody (Ab) have been used recently as biomarkers for RA diagnosis. In particular, the anti-CCP Ab has been reported to be highly specific and sensitive for RA (24). Autoantibody production is related strongly with the *HLA* region (25).

In this study, we have conducted a comprehensive case–control study of the *HLA* association with RA and the presence of autoantibodies by genotyping the six classical *HLA* loci and reconstructing their haplotypes in Japanese RA patients and controls.

Materials and methods

Subjects

A total of 622 Japanese RA patients were enrolled from among outpatients of the Division of Rheumatology, Tokai University Hospital (427 cases), and Division of Rheumatology, Keio University Hospital (195 cases). All RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR) (26). Two rheumatologists independently evaluated all cases as a blind analysis of clinical information. Among 622 cases, 488 (82.6%) were anti-CCP Ab positive, 103 (17.4%) were anti-CCP Ab negative and the serology for 31 cases was unknown (Table 1).

The 427 RA patients from Tokai University Hospital were divided into three subsets based on the number of joints with erosion and the time course of erosion (27): mutilating disease (MUD), more erosive disease (MES) and least erosive disease (LES). Almost all the joints were extensively damaged in the MUD subset. In MES, in addition to the involvement of peripheral smaller joints in patients, the larger axial joints such as knees, hips, shoulders and elbows were also involved. In LES, erosive articular changes were primarily limited to the peripheral smaller joints during the course of disease. The order of Sharp score is expected to be MUD > MES > LES. The MUD group was combined with the MES group for more reliable statistical analyses because of the small number of MUD cases (only 17 MUD cases of the total 427 RA

Table 1 Case and control subjects used in this study, number of males (M) and females (F) in each subset and anti-CCP antibody positivity

	Cases											Controls		
	Group 1 ($n = 427$)						Group 2 ($n = 195$)		Total ($n = 622$)			($n = 1037$)		
	MUD+MES		LES		Uk									
	M	F	M	F	M	F	M	F	M	F	Overall	M	F	Overall
n	47	240	27	108	0	5	46	149	120	502	622	543	494	1,037
Age	63.6	62.5	60.4	57.8	—	62.5	62.0	57.8	62.3	59.7	60.2	55.4	53.0	54.3
SD	13.8	12.6	13.5	13.6	—	11.4	15.4	14.2	14.6	13.6	13.8	10.4	10.2	10.4
Anti-CCP Ab														
(+)	41	209	18	83	0	4	27	106	86	402	488	3	10	13
(−)	6	31	9	23	0	1	12	21	27	76	103	540	484	1024
Uk	0	0	0	2	0	0	7	22	7	24	31	0	0	0

Ab, antibody; CCP, cyclic citrullinated peptides; LES, least erosive disease; MES, more erosive disease; MUD, mutilating disease; uk SD, standard deviation; UK, not known.

© 2012 John Wiley & Sons A/S

cases). The number of each subset and percentage of anti-CCP positive cases are as follows: MUD; 17 cases; 94.1% (anti-CCP positive), MES; 270; 86.7%, LES; 135; 75.9%. Duration times of disease for all cases from Tokai University Hospital were more than 5 years as previously described (27). The breakdown of cases with and without autoantibody positivity is summarized in Table 1.

A total of 1037 unrelated healthy Japanese control subjects without a family history of RA were recruited from among visitors to the Health Evaluation and Promotion Center of Tokai University Hospital. Details on RA symptoms and family history were obtained from control subjects at the time of the informed consent. The number of anti-CCP Ab-positive subjects among the 1037 controls was 13 (1.3%). All of the 1037 controls used had no family history.

All subjects gave written informed consent for genetic screening. Ethical approvals for this study were obtained from the ethical committee of Tokai University School of Medicine and from the ethical committee of Keio University School of Medicine.

HLA typing

DNA samples were extracted from peripheral blood using the DNA extraction kit (Genomix, SRL, Tokyo, Japan). We analyzed six *HLA* loci (*HLA-A*, *–B*, *–C*, *-DRB1*, *-DQB1* and *-DPB1*) using the Luminex assay system and HLA typing kits (WAKFlow HLA Typing kits, Wakunaga, Osaka, Japan or LABType SSO, One Lambda, Canoga Park, CA). The observed allele frequencies showed good concordance with the reported allele frequencies by the Central Bone Marrow Data Center, Japanese Red Cross Society (http://www.bmdc.jrc.or.jp/stat.html). The *P*-values obtained by the Fisher's exact test in the comparison between our data and the data of Japanese Red Cross Society were as follows: 0.782 for *HLA-A*, 0.719 for *HLA-B*, 0.067 for *HLA-C* and 0.743 for *HLA-DRB1*.

Serologic assessment

The anti-CCP antibody (autoantibody) status of the cases and controls was determined using the MESACUP-2 test CCP (Medical & Biological Laboratories, Nagoya, Japan) with a cutoff value of 4.5 U/ml. Autoantibody was measured at the time of diagnosis without treatment. In negative cases, we measured autoantibody again one year after the start of treatment.

Statistical analyses

Fisher's exact test using R-software was performed to analyze the significant differences between groups and to obtain *P*-values, odds ratios (ORs) and 95% confidence intervals (95% CI). Bonferroni correction was made by multiplying the *P*-value with the number of *HLA* alleles observed in this study: 23 for *HLA-A*, 41 for *HLA-B*, 21 for *HLA-C*, 33 for *HLA-DRB1*, 14 for *HLA-DQB1* and 19 for *HLA-DPB1*. The Cochran–Armitage test for trends was performed using R-software. *HLA* haplotypes were estimated using PHASE version 2.1.1 (28).

Results

Association between HLA alleles and RA stratified by the presence or absence of autoantibodies

In comparison with controls the *HLA* allele frequencies of some loci were significantly increased or decreased in RA patients. The anti-CCP Ab-positive group showed significant increases or decreases of particular *HLA* alleles in the RA patients (Table 2) in agreement with a previous report on the association between the SE and anti-CCP Ab positivity in the Dutch population (29). *HLA-DR3* was associated previously with the anti-CCP Ab-negative RA (30), However, no association was detected in our study because only one RA patient had *HLA-DRB1*03:01* and this allele has a low frequency of 0.13% in the Japanese according to the data of the Central Bone Marrow Data Center, Japanese Red Cross Society (http://www.bmdc.jrc.or.jp/stat.html). We also found no association between anti-CCP Ab-negative RA and *DRB1*09:01* ($P = 1.00$, OR = 0.99, 95% CI = 0.64–1.49) or *DRB1*12:01* ($P = 0.023$, $pc = 0.54$, OR = 2.04, 95% CI = 1.07–3.66), although such associations in Japanese was previously reported (31, 32). Moreover, the sero-negative RA patients were not associated with any SE groups similarly to the results of a previous report (33). Therefore, all further analyses were conducted on the association between *HLA* alleles and RA using only the anti-CCP Ab-positive patients.

Association between *HLA-DRB1* alleles and anti-CCP Ab-positive RA

The significant ($P < 0.05$) RA-susceptible *DRB1* alleles were *DRB1*04:05*, **04:01* and **10:01* and the significant ($P < 0.05$) RA-protective *DRB1* alleles were *DRB1*13:02*, **14:05* and **08:02* (Table 2). These findings in Japanese showed good concordance with previous reports. However, we could not detect the association between *DRB1*09:01* and anti-CCP Ab-positive RA (allele frequencies, 17.0 % in RA patient, 15.2% in control; $P = 0.202$, OR = 1.14, 95% CI = 0.93–1.41), although *HLA-DRB1*09:01* showed a low *P*-value (3.76e-09) in Koreans (34).

Association between *HLA-DPB1* haplotypes and anti-CCP Ab-positive RA

There were no RA-specific four HLA-loci or six HLA-loci haplotypes with more than 0.5% haplotype frequency

© 2012 John Wiley & Sons A/S