

Brief report

Defective circulating CD25 regulatory T cells in patients with chronic immune thrombocytopenic purpura

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Immune thrombocytopenic purpura (ITP) is characterized by the presence of anti-platelet autoantibodies as a result of loss of tolerance. CD4⁺CD25⁺ regulatory T cells (Tregs) are important for maintenance of peripheral tolerance. Decreased levels of peripheral Tregs in patients with ITP have been reported. To test whether inefficient production or reduced immunosuppressive activity of Tregs contributes

to loss of tolerance in patients with chronic ITP, we investigated the frequency and function of their circulating CD4⁺CD25^{hi} Tregs. We found a comparable frequency of circulating CD4⁺CD25^{hi}Foxp3⁺ Tregs in patients and controls (n = 16, P > .05). However, sorted CD4⁺CD25^{hi} cells from patients with chronic ITP (n = 13) had a 2-fold reduction of in vitro immunosuppressive

activity compared with controls (n = 10, P < .05). The impaired suppression was specific to Tregs as shown by cross-mixing experiments with T cells from controls. These data suggest that functional defects in Tregs contribute to breakdown of self-tolerance in patients with chronic ITP. (Blood. 2008;112:1325-1328)

Introduction

Immune thrombocytopenic purpura (ITP) is a bleeding disorder characterized by production of autoreactive antibodies to platelet antigens, resulting in both accelerated destruction of platelets and reduced platelet production.¹ While healthy individuals harbor platelet-specific autoreactive T cells that are tolerized in the periphery,² patients with ITP possess activated platelet autoreactive T cells and cytokine imbalance,³⁻⁷ suggesting loss of peripheral tolerance in ITP patients. CD4⁺ regulatory T cells (Tregs) play an important role in maintenance of peripheral tolerance and are characterized by the expression of the CD25 surface marker and the transcription factor forkhead box protein 3 (Foxp3), making up 5% to 10% of the normal CD4⁺ T-cell population.⁸

Different populations of Tregs have been described, including naturally occurring and inducible Tregs.⁹ The former are thymically derived and suppress general autoreactive responses under noninflammatory conditions, although they can also become activated and expand in an antigen-specific manner.¹⁰ Inducible Tregs are generated in the periphery through exposure to antigen, but once activated are thought to mediate suppressive activity against other antigens by the local release of specific cytokines.¹¹ Several reports have demonstrated Treg alterations in a number of autoimmune diseases.¹²⁻¹⁶ These reports suggest that circulating Treg frequency and/or function may be used as a marker for evaluating autoimmune status in patients. Recent studies in patients with ITP have shown reduced levels of Foxp3 mRNA¹⁷ and protein¹⁸ in circulating mononuclear cells and abnormal Treg function in spleen biopsies.¹⁹ These studies indicate that deficiency in generation and/or defective functions of Tregs may contribute to loss of immunologic self-tolerance in patients with ITP. To test the

hypothesis that the pathogenesis of chronic ITP may be related to the levels or function of circulating peripheral Tregs, we examined the frequency of Tregs in peripheral blood mononuclear cells (PBMCs) from patients with chronic ITP by flow cytometry and performed in vitro assays to assess the immunosuppressive effect of Tregs on CD4⁺ T-cell proliferation.

Methods

Subjects

We enrolled 17 patients with chronic refractory ITP (Table 1) and 16 age-matched and closely age-matched healthy donors in this study, and informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Boards of the Weill Medical College of Cornell University and of the New York Blood Center (NYBC).

Cell staining and purification

Within 2 hours of collection, whole blood was stained with anti-CD4 and anti-CD25 (both from BD Pharmingen, San Diego, CA) followed by Foxp3 staining (clone PCH101; eBioscience, San Diego, CA) according to the manufacturer's instructions and analyzed by flow cytometry (FACSCanto cytometer with FACSDiva software; BD Biosciences, San Jose, CA). Due to the lack of a Treg cell-specific surface marker, isolation of human Treg products has relied on using the CD4⁺CD25^{hi} T-cell population.²⁰ To purify Tregs, CD4⁺ cells were first enriched by positive selection (Miltenyi Biotech, Auburn, CA) of PBMCs isolated by Ficoll-Paque gradient centrifugation. After staining, CD4⁺CD25^{hi} and CD4⁺CD25⁻ T cells were separated (> 94% purity) on a MoFlo (Beckman, Hialeah, FL) cell sorter.

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Table 1. Demographic and clinical characteristics of patients with chronic ITP and correlation with frequency and levels of Foxp3⁺ in CD4⁺CD25^{hi} T-cell population

Patient no.	Age, y	Sex	Splenectomy*	PLT, ×10 ⁹ /L	Disease duration, y	%Foxp3 ⁺ /CD25 ^{hi}	%Foxp3 ⁺ reactivity	Treatment type at time of collection
1	60	M	Y	68	7	9	75.6	Steroidal (IVIg)
2	50	F	N	229	12	7.6	86.1	Nonsteroidal (thrombopoietic)
3†	14	M	N	105	4	7.1	73.3	Nonsteroidal (IVIg)
4	62	F	Y	586	6	6.4	85.7	Steroidal (IVIg)
5†	58	F	N	92	5	6.4	91.1	Steroidal (IVIg)
6	50	M	Y	61	8	5.4	89.2	Nonsteroidal (thrombopoietic)
7	83	F	Y	120	13	5.3	81.1	Nonsteroidal (thrombopoietic)
8	77	M	Y	26	7	5.3	82.2	Nonsteroidal (Syk inhibitor)
9†	45	F	N	58	1.7	4.5	86.1	Nonsteroidal (Syk inhibitor)
10	44	F	Y	41	2.5	4.3	82.9	Nonsteroidal (thrombopoietic)
11†	52	F	Y	11	48	4.3	74.5	Nonsteroidal (thrombopoietic)
12	53	M	Y	15	18	3	82.2	Nonsteroidal (thrombopoietic)
13	46	F	N	7	12	2.7	78.1	Nonsteroidal (Syk inhibitor)
14	56	F	Y	280	10	2.6	84.8	Nonsteroidal (Syk inhibitor)
15	71	M	N	4	10	2.5	57.4	Nonsteroidal (thrombopoietic)
16	30	F	Y	9.5	16	2.4	54.0	Nonsteroidal (Syk inhibitor)
17	48	M	N	21	1.3	ND	ND	Nonsteroidal (WinRho)

Patients 3 and 16 identified themselves as Hispanic. The rest identified themselves as white. Intravenous immunoglobulin (IVIg) treatments with or without steroids were indicated and WinRho in patient 17 were administered at least 7 days prior to collection of blood for analysis. "Syk inhibitor" and "thrombopoietic" refer to Syk kinase inhibitor and thrombopoietic agents, respectively, and are both investigational drugs.

PLT indicates platelets; and ND, not determined.

*Y indicates yes; N, no, for patients who had or had undergone splenectomy.

†Patients (3, 5, 9, and 11) for whom Treg functional activity was not determined.

Only cells with a slightly lower CD4 expression level within the CD4⁺CD25^{hi} population were sorted as previously reported.²⁰ T cell-depleted antigen presenting cells (APCs) were obtained by negative selection (Miltenyi Biotech) of allogeneic PBMCs and irradiation.

Proliferation assay

CD4⁺CD25⁻ and CD4⁺CD25^{hi} T cells (5×10^4 cells/well) were cultured in duplicates or triplicates alone or together at various ratios in the presence of plate-bound anti-CD3 (clone UCHT1, 0.1 μg/mL; BD Biosciences) together with 10^5 allogeneic APCs/well. After 5 days, 1 μCi (0.037 MBq) [³H]thymidine was added to each well and after 16 hours, [³H]thymidine incorporation was measured by scintillation counting (PerkinElmer, Waltham, MA). Percentage inhibition was determined as $1 - (\text{cpm incorporated in the coculture}) / (\text{cpm of responder cells alone}) \times 100$.

Statistical analysis

An ANOVA test was used to evaluate possible differences in the Treg frequency and function between patients and healthy donors.

Results and discussion

The mean frequency of Foxp3⁺CD25^{hi} in the CD4⁺ population, which is characteristic of the human PBMC Treg phenotype,²¹⁻²³ was 4.9% ($\pm 0.5\%$) in the patient group ($n = 16$, Table 1) and 5.1% ($\pm 0.3\%$) in controls ($n = 16$, $P = .7$, Figure 1A,B). There was a trend for levels of Foxp3⁺ reactivity to be reduced in patients compared with controls ($78.9\% \pm 2.6\%$ vs $85.7\% \pm 2.4\%$, Figure 1C,D) as in previous studies,^{17,18} but the differences did not quite reach statistical significance.

In 13 patients (Table 1) and 10 controls, we examined the immunosuppressive effects of Tregs on proliferation of autologous CD4⁺CD25⁻ cells. Sorted CD4⁺CD25^{hi} T cells from both patients and controls were equally hyporesponsive to polyclonal stimulation provided by anti-CD3 and allogeneic APCs (mean of ³H incorporation for patients $991 \text{ cpm} \pm 116 \text{ cpm}$ and controls

$917 \text{ cpm} \pm 218 \text{ cpm}$; $P > .7$), indicating that patient CD4⁺CD25^{hi} T cells do exhibit the characteristic anergic Treg phenotype.²⁴ In contrast, sorted CD4⁺CD25⁻ cells from both patients and controls responded, albeit similarly, to stimulation (mean of ³H incorporation: for patients, $10\,860 \text{ cpm} \pm 4955 \text{ cpm}$ and controls, $14\,507 \text{ cpm} \pm 6100 \text{ cpm}$; $P > .9$).

The proliferative responses of CD4⁺CD25⁻ cells from healthy controls upon coculture with autologous CD4⁺CD25^{hi} cells at a 1:1 ratio were inhibited by 74%, and this suppression was reduced with decreasing suppressor-responder ratios (44% and 21% inhibition at 1:4 and 1:16, respectively, Figure 1E), consistent with previous reports.^{21,24} In contrast, CD4⁺CD25^{hi} T cells from patients were less effective suppressors, inhibiting the proliferation of autologous CD4⁺CD25⁻ cells with half the efficiency (44%, 22%, and 5% inhibition at 1:1, 1:4, and 1:16 ratios, respectively, $P < .05$, Figure 1E).

The reduced regulatory function in patients with chronic ITP could be ascribed to a decrease in CD4⁺CD25^{hi} Treg function or to refractoriness of CD4⁺CD25⁻ cells to suppression. Cross-mixing experiments were performed in which patient and control regulatory CD4⁺CD25^{hi} cells were cocultured with the autologous and the converse CD4⁺CD25⁻ cells from either controls or patients. We found that patient CD4⁺CD25^{hi} Tregs ($n = 5$) could not effectively inhibit proliferation of CD4⁺CD25⁻ cells from either patients or healthy controls (Figure 1F). In contrast, CD4⁺CD25^{hi} Tregs from controls ($n = 3$) suppressed the proliferation of CD4⁺CD25⁻ cells from both patients and controls to a similar degree (Figure 1G). We have therefore demonstrated that circulating CD4⁺CD25^{hi} T cells are functionally defective in patients with chronic ITP instead of resistant to suppression by the responder patient cells. Polyclonal non-antigen-specific stimuli were used to assess in vitro Treg function, and the relative contribution of naturally occurring and inducible Tregs to suppression was not determined. Nevertheless, the data, while correlative, indicate an overall impaired Treg function in patients with chronic ITP.

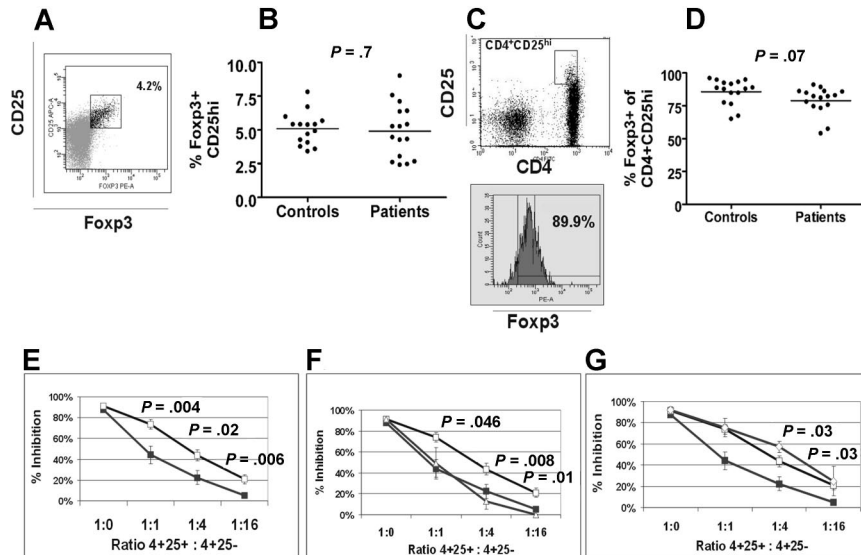


Figure 1. Treg frequency and function in patients with chronic ITP. (A) Representative dot plot of CD25 and Fxp3 reactivity of gated CD4⁺ T-cell population in PBMCs. Isotype control for anti-Fxp3 antibody was used to set the gates for calculating the percentage of the Fxp3⁺CD25^{hi} cells within the CD4⁺ subset. (B) The percentages of Fxp3⁺CD25^{hi} cells in controls and patients with chronic ITP as calculated by the gating strategy shown in panel A. (C) Representative dot plot of CD4 and CD25 in total PBMCs with the box showing the CD4⁺CD25^{hi} population. Below the dot plot is the histogram showing the levels of Fxp3 reactivity in the CD4⁺CD25^{hi} subset expressed as percentage. The gating was set using isotype control for anti-Fxp3 antibody. (D) The levels of Fxp3 reactivity in controls and patients shown as percentages based on gating strategy in panel C. Horizontal bars represent the medians in panels B and D. (E) The mean percentage inhibition of the proliferative response of stimulated CD4⁺CD25⁻ cells by autologous CD4⁺CD25^{hi} cells from 13 of the chronic ITP patients (■) and 10 healthy controls (□) was calculated ("Proliferation assay"). CD4⁺CD25^{hi} and CD4⁺CD25⁻ sorted cells were stimulated with plate-bound 0.1 μg/mL anti-CD3 antibodies and allogeneic accessory cells, alone or cocultured at various suppressor-responder ratios (1:1, 1:4, and 1:16). Addition of CD4⁺CD25^{hi} cells at 1:1 ratios to CD4⁺CD25⁻ cells inhibited proliferative responses and decreasing the number of CD4⁺CD25^{hi} cells resulted in less inhibition of proliferation. For comparison, percentage inhibition of proliferation of CD4⁺CD25^{hi} when stimulated alone (1:0) is also shown. Regulatory T cells from patients with chronic ITP showed significantly less suppressor activity. Error bars depict standard error of the mean. (F) CD4⁺CD25⁻ responder T cells from healthy donors (n = 5) were cocultured with CD4⁺CD25^{hi} cells from patients with chronic ITP (n = 5) using the same conditions as in panel E, and mean percentage inhibition was calculated for the various responder-suppressor ratios (Δ). For comparison, the mean percentage inhibition of autologous proliferative responses of patients (n = 13) and controls (n = 10) from panel E is shown. The P values reflect the difference between the suppression of proliferation of CD4⁺CD25⁻ responder T cells from healthy donors using CD4⁺CD25^{hi} from healthy controls versus CD4⁺CD25^{hi} from patients. (G) Responder cells from patients with chronic ITP (n = 3) were cocultured with CD4⁺CD25^{hi} from healthy controls (n = 3) as in panel E, and mean percentage inhibition was calculated (◇). For comparison, the mean percentage inhibition of autologous proliferative responses from panel E is shown. The P values reflect the difference between the suppression of proliferation of CD4⁺CD25⁻ responder T cells from patients with chronic ITP using CD4⁺CD25^{hi} from healthy controls versus autologous patient CD4⁺CD25^{hi}.

Although it remains to be tested, failure to maintain immune suppression may explain the reported platelet autoantigen-specific T-cell proliferative responses and the proinflammatory T helper 1 phenotype in ITP patients.²⁵ The patients were on various treatment regimens including nonimmunogenic thrombopoietic agents, and yet their Treg function was impaired regardless of treatment type (Table 1). Because the patients had refractory chronic ITP, they could not be evaluated "off treatment." To determine whether there is an association of Treg function with age, sex, platelet count, prior splenectomy, ITP duration, or treatment regimen would require much larger numbers of patients. The defect in Treg function remains to be defined but may include perturbations in cell-cell interactions and/or cytokine signaling, both implicated as potential mechanisms underlying Treg-mediated immunosuppression.⁸ Overall, the data suggest that the demonstrated dysfunction of Tregs contributes to loss of tolerance in chronic ITP. These findings raise the possibility that Tregs may be a therapeutic target in these patients.

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Authorship

Contribution: J.Y. and S.H. performed research, and analyzed and interpreted data; V.P. selected and recruited patients, analyzed data, and wrote the paper; J.L. recruited patients and analyzed data; Y.Y. performed research; J.B.B. designed the research, selected and recruited patients, and wrote the paper; and K.Y. designed, directed, and performed research, and wrote the paper.

Conflict-of-interest disclosure: J.B.B. receives research grants from Amgen, Biogen-IDEC, Cangene, Genentech, GlaxoSmithKline (GSK), and Sysmex; receives lecture fees from Baxter; and receives consulting fees from Amgen, Symphogen, GSK, and Baxter; has participated in Advisory Boards for Amgen, GSK, Ligand, and Baxter; and has equity ownership in Amgen and GSK. The other authors declare no competing financial interests.

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