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+CD25hi Tregs. We found a comparable frequency of circulating CD4+CD25hiFoxp3+ Tregs in patients and controls (n = 16, P > .05). However, sorted CD4+CD25hi 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 autoantibodies to platelet antigens, resulting in both accelerated destruction of platelets and reduced platelet production. While healthy individuals harbor platelet-specific autoantibotic T cells that are tolerized in the periphery, patients with ITP possess activated platelet autoimmune T cells and cytokine imbalance, 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.

Different populations of Tregs have been described, including naturally occurring and inducible Tregs. 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. 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. Several reports have demonstrated Treg alterations in a number of autoimmune diseases. 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 mRNA and protein in circulating mononuclear cells and abnormal Treg function in spleen biopsies. 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 (FACSQuant cytometer with FACSDiva software; BD Biosciences, San Jose, CA). Due to the lack of a Treg cell–specific surface marker, isolation of human Treg cells was performed in vitro. T cells were separated (> 94% purity) on a MoFlo (Beckman, Hialeah, FL) cell sorter.


*J.Y. and S.H. contributed equally to this work.

An Inside Blood analysis of this article appears at the front of this issue.

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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+CD25hi T-cell population

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, y</th>
<th>Sex</th>
<th>Splenectomy*</th>
<th>PLT, ×10^12/L</th>
<th>Disease duration, y</th>
<th>%Foxp3+/CD25hi</th>
<th>%Foxp3+ reactivity</th>
<th>Treatment type at time of collection</th>
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<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>M</td>
<td>Y</td>
<td>68</td>
<td>7</td>
<td>9</td>
<td>75.6</td>
<td>Steroidal (IVIG)</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>F</td>
<td>N</td>
<td>229</td>
<td>12</td>
<td>7.6</td>
<td>86.1</td>
<td>Nonsteroidal (thrombopoietic)</td>
</tr>
<tr>
<td>3†</td>
<td>14</td>
<td>M</td>
<td>N</td>
<td>105</td>
<td>4</td>
<td>7.1</td>
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<td>Nonsteroidal (IVIG)</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>F</td>
<td>Y</td>
<td>586</td>
<td>6</td>
<td>6.4</td>
<td>85.7</td>
<td>Steroidal (IVIG)</td>
</tr>
<tr>
<td>5†</td>
<td>58</td>
<td>F</td>
<td>N</td>
<td>92</td>
<td>5</td>
<td>6.4</td>
<td>91.1</td>
<td>Steroidal (IVIG)</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>M</td>
<td>Y</td>
<td>61</td>
<td>8</td>
<td>5.4</td>
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<td>Y</td>
<td>120</td>
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<tr>
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<td>77</td>
<td>M</td>
<td>Y</td>
<td>26</td>
<td>7</td>
<td>5.3</td>
<td>82.2</td>
<td>Nonsteroidal (Syk inhibitor)</td>
</tr>
<tr>
<td>9†</td>
<td>45</td>
<td>F</td>
<td>N</td>
<td>58</td>
<td>1.7</td>
<td>4.5</td>
<td>86.1</td>
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</tr>
<tr>
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<td>F</td>
<td>Y</td>
<td>41</td>
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<td>4.3</td>
<td>82.9</td>
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</tr>
<tr>
<td>11†</td>
<td>52</td>
<td>F</td>
<td>Y</td>
<td>11</td>
<td>48</td>
<td>4.3</td>
<td>74.5</td>
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<tr>
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<td>53</td>
<td>M</td>
<td>Y</td>
<td>15</td>
<td>18</td>
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<td>82.2</td>
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<tr>
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<td>46</td>
<td>F</td>
<td>N</td>
<td>7</td>
<td>12</td>
<td>2.7</td>
<td>78.1</td>
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</tr>
<tr>
<td>14</td>
<td>56</td>
<td>F</td>
<td>Y</td>
<td>280</td>
<td>10</td>
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<td>84.8</td>
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<tr>
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<td>71</td>
<td>M</td>
<td>N</td>
<td>4</td>
<td>10</td>
<td>2.5</td>
<td>57.4</td>
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</tr>
<tr>
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<td>30</td>
<td>F</td>
<td>N</td>
<td>9.5</td>
<td>16</td>
<td>2.4</td>
<td>54.0</td>
<td>Nonsteroidal (Syk inhibitor)</td>
</tr>
<tr>
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<td>M</td>
<td>N</td>
<td>21</td>
<td>1.3</td>
<td>ND</td>
<td>ND</td>
<td>Nonsteroidal (WinRho)</td>
</tr>
</tbody>
</table>

Patients 3 and 16 identified themselves as Hispanic. The rest identified themselves as white. Intravenous immunoglobulin (IVIG) treatments with or without steroids where 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. PLT indicates platelets; and ND, not determined.

Only cells with a slightly lower CD4 expression level within the CD4+CD25hi 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+CD25hi T cells (5 × 10^6 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[1]thymidine was added to each well and after 16 hours, ^3H[1]thymidine incorporation was measured by scintillation counting (PerkinElmer, Waltham, MA). Percentage inhibition was determined as 1 – (cpm incorporated in the coculture/cpm of responder cells alone) × 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+CD25hi in the CD4+ population, which is characteristic of the human PBMC Treg phenotype,21-23 was 4.9% (± 0.5%) in the patient group (n = 16, Table 1) and 5.1% (± 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% ± 2.6% vs 85.7% ± 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+CD25hi T cells from both patients and controls were equally hyporesponsive to polyclonal stimulation provided by anti-CD3 and allogeneic APCs (mean of ^3H[1] incorporation for patients 991 cpm ± 116 cpm and controls 917 cpm ± 218 cpm; P > .7), indicating that patient CD4+CD25hi 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 cpm ± 4955 cpm and controls, 14 507 cpm ± 6100 cpm; P > .9).

The proliferative responses of CD4+CD25− cells from healthy controls upon coculture with autologous CD4+CD25hi 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+CD25hi 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+CD25hi Treg function or to refractoriness of CD4+CD25− cells to suppression. Cross-mixing experiments were performed in which patient and control regulatory CD4+CD25hi cells were cocultured with the autologous and the converse CD4+CD25− cells from either controls or patients. We found that patient CD4+CD25hi Tregs (n = 5) could not effectively inhibit proliferation of CD4+CD25− cells from either patients or healthy controls (Figure 1F). In contrast, CD4+CD25hi 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+CD25hi 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.
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.\textsuperscript{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.\textsuperscript{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

Consultation- and interpretation 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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References


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