Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease

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Chronic graft-versus-host disease (GVHD) is the most common cause of poor long-term outcomes after allogeneic bone marrow transplantation (BMT), but the pathophysiology of chronic GVHD still remains poorly understood. We tested the hypothesis that the impaired thymic negative selection of the recipients will permit the emergence of pathogenic T cells that cause chronic GVHD. Lethally irradiated C3H/HeN (H-2k) recipients were reconstituted with T-cell-depleted bone marrow cells from major histocompatibility complex [MHC] class II–deficient (H2-Ab1–/) B6 (H-2b) mice. These mice developed diseases that showed all of the clinical and histopathological features of human chronic GVHD. Thymectomy prevented chronic GVHD, thus confirming the causal association of the thymus. CD4+ T cells isolated from chronic GVHD mice were primarily donor reactive, and adoptive transfer of CD4+ T cells generated in these mice caused chronic GVHD in C3H/HeN mice in the presence of B6-derived antigen-presenting cells. Our results demonstrate for the first time that T cells that escape from negative thymic selection could cause chronic GVHD after allogeneic BMT. These results also suggest that self-reactivity of donor T cells plays a role in this chronic GVHD, and improvement in the thymic function may have a potential to decrease chronic GVHD. (Blood. 2007;109:1756-1764)

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is considered to be an effective therapy for a variety of malignant and nonmalignant diseases of the lymphohematopoietic system. Chronic graft-versus-host disease (GVHD) is a complex multiorgan disorder with features of autoimmunity and immunodeficiency. Chronic GVHD remains the major cause of late death and morbidity after allogeneic HSCT. Unfortunately, despite success in the prevention of acute GVHD by the introduction of cyclosporine, the incidence of chronic GVHD has not decreased. Its incidence is increasing with the greater use of unrelated or HLA-mismatched donors, older donors and recipients, donor lymphocyte infusions, and peripheral blood stem cell transplantation. These observations suggest that chronic GVHD is not simply a continuation of acute GVHD, and therefore novel strategies are required to prevent chronic GVHD. Although progress has been made in understanding the pathophysiology of acute GVHD, the basic pathophysiology of chronic GVHD remains poorly understood.

The existing experimental models of chronic GVHD have certain limitations. For example, the most commonly used mouse model of chronic GVHD is induced by the injection of parental (P) cells into nonirradiated F1 recipients.11 This model mimics systemic lupus erythematosus (SLE) with B-cell expansion, splenomegaly, autoantibody production, and glomerulonephritis as a result of a cognate interaction between donor CD4+ T cells and host B cells.12,13 Another murine model, B10.D2 (H-2d) into an irradiated BALB/c (H-2b) model, mimics human chronic GVHD with fibrosis of the skin and exocrine glands and hepatic and pulmonary involvement.14,15 In these models, mature T cells infused are responsible for the induction of chronic GVHD. A recently developed chronic GVHD model that was made by the transplantation of DBA/2 spleen cells into sublethally irradiated BALB/c mice also leads mice to develop SLE-like features that require both donor CD4+ T cells and B cells.16 In this model, host thymus is not required for the induction of chronic GVHD.

T-cell repopulation following HSCT results from both thymic-dependent and thymic-independent pathways. It is now obvious that the thymic-independent peripheral expansion of mature T cells is responsible for the development of acute GVHD because T-cell depletion (TCD) of the donor bone marrow (BM) reduces rates of acute GVHD both in mice and humans.18,19 The infusion of mature donor CD4+ T cells is also responsible for chronic GVHD in P→F1, DBA/2→BALB/c, and B10.D2→BALB/c models.11,15,16 However, it has been postulated that donor-derived T cells generated from hematopoietic stem cells via the recipient’s thymus could also cause chronic GVHD.20,22 While it has been clear that ex vivo TCD from the donor BM reduces the incidence of acute GVHD,18,19 the effect of TCD on chronic GVHD has been less well delineated. The first randomized study comparing TCD–BM transplantation (BMT) with T-cell replete BMT found that TCD of the donor BM reduces rates of acute GVHD but not chronic GVHD,23 thus supporting the hypothesis that thymic-dependent T cells play a role in mediating chronic GVHD.

Within the thymus, T cells undergo both positive and negative selection, thus resulting in the elimination of self-reactive cells.
Positive selection is mediated by the thymic cortical epithelium, while negative selection via clonal deletion is mediated mainly by thymic dendritic cells (DCs). Recent evidence clearly demonstrates that thymopoiesis continues even in adult recipients after allogeneic BMT. The thymus is damaged by prior chemotherapy, conditioning regimen, acute GVHD, and age-related atrophy. Experimental data indicate that such thymic damage results in a loss of thymic negative selection. We therefore hypothesized that chronic GVHD could thus be the result of autoreactive T cells that escape negative selection in the damaged thymus. In fact, the incidence of chronic GVHD is lower in pediatric patients, whose thymic function is better than in adults. We tested the hypothesis that an impaired negative selection due to the loss of major histocompatibility complex (MHC) class II expression in thymic DCs causes chronic GVHD. We found that these mice developed lethal disease similar to human chronic GVHD after allogeneic BMT.

Materials and methods

Mice

Female C57BL6 (B6: H-2b), C3H/HeN (C3H: H-2k), and BALB/c (H-2d) mice were purchased from Charles River Japan (Yokohama, Japan). B6-background MHC class II–deficient H2-Ab1−/− mice (B6.129-Ahbpmt N12) were from Taconic (Germantown, NY). The age range of the mice was from 8 to 16 weeks. The mice were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 weeks after transplantation. All experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources at Okayama University and Kyushu University.

BMT

C3H mice were exposed to 13 Gy total body irradiation (TBI; x-ray) split into 2 doses and then injected intravenously with 5 × 10^6 TCD-BM cells from wild-type B6 (WT) or H2-Ab1−/− mice on day 0. BALB/c mice were exposed to 9.5 Gy TBI. TCD-BM was performed using CD90 microbeads and the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). In some experiments, C3H mice were thymectomized prior to BMT as described. Hybridomas secreting anti-CD8 monoclonal antibodies (mAbs) and anti-CD25 mAbs were obtained from American Type Culture Collection (Manassas, VA). For in vivo depletion of CD8+ cells, mice were injected intraperitoneally with 2 mg anti-CD8 mAb on day 7 after BMT and 1 mg every week thereafter. For CD25 depletion, mice were injected intravenously with 0.5 mg anti-CD25 mAb on day 0 and every 10 days thereafter as described.

Assessment of GVHD

The survival after BMT was monitored daily, and weight changes were assessed weekly. The degree of clinically acute GVHD was assessed weekly by a scoring system that sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described. Samples of skin, liver, and intestine were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, slide mounted, and stained with hematoxylin and eosin. Histological images were composed using Adobe Illustrator 10. The severity of chronic GVHD of the skin was assessed by a scoring system that incorporates 5 parameters: epidermis atrophy, increased collagen density in dermis, fat atrophy, inflammation, and follicle atrophy. The slides were graded from 0 to 2 for each parameter, and pathologic scores were subsequently generated by the summation of the 5 criteria scores (maximum index, 10). Chronic GVHD was defined as sclerodermatous skin change and/or one of the following pathologic features: skin sclerotic change, ductopenia/portal fibrosis in the liver, or destruction/fibrosis of the salivary glands. Acute GVHD was defined as the absence of chronic GVHD manifestations but the presence of villous atrophy with crypt cell apoptosis in the intestine.

Adoptive transfer experiments

Splenocytes were isolated from the recipient mice 6 to 11 weeks after TCD-BMT. CD4+ T cells were negatively selected from splenocytes by depleting CD8+, DX5+, CD11b+, Ter-119+, and B220+ cells using the AutoMACS system. Purity of the CD4+ T-cell subset was more than 85%, and contamination of CD8+ cells was less than 3%. A total of 1 × 10^7 CD4+ T cells from either [WT → C3H] or [H2-Ab1−/− → C3H] mice along with 5 × 10^6 TCD-BM cells from B6 or C3H mice were injected intravenously into C3H mice following 13 Gy TBI.

Cell culture

Thymic DCs were isolated as described. BM-derived DCs were generated by culturing BM cells in the presence of 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20 ng/mL interleukin 4 (IL-4) (Peprotech, London, United Kingdom) for 4 days. Splenic CD4+ T cells were cultured with irradiated (20 Gy) DCs. Seventy-two hours after the initiation of culture, proliferation was determined by a thymidine uptake assay as described.

Flow cytometric analysis

The mAbs used were FITC-, PE-, or allophycocyanin-conjugated anti-mouse TCRβ, CD4, CD8α, CD25, H-2Kb, H2-Kk, I-Aβ (BD Pharmingen, San Diego, CA), and Foxp3 (eBioscience, San Diego, CA). The cells were stained and analyzed as described. Dead cells were determined as 7-amino-actinomycin D (BD Pharmingen)–positive cells. At least 5000 live events were acquired for the analysis. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, CD4+ T cells negatively selected from the recipient mice were stained in PBS with 1 μM CFSE purchased from Molecular Probes (Eugene, OR). These CFSE-labeled cells were stimulated with irradiated splenocytes in culture. Four days later, cell division was assessed as dilution of CFSE in CD4+ T cells.

Statistical analysis

The survival curves were plotted using Kaplan-Meier estimates. The Mann-Whitney U test was used for the statistical analysis of the in vitro data and the pathology scores, while the Mantel-Cox log-rank test was used to compare survival curves. P values less than .05 were considered statistically significant.

Results

Allogeneic TCD-BMT from MHC class II–deficient donors caused chronic GVHD

Allogeneic transplantation of TCD-BM does not induce acute or chronic GVHD in mice. This may be due to a near normal thymic function after TBI in mice. We first examined whether impaired thymic negative selection could allow the emergence of donor-
host-reactive T cells after allogeneic TCD-BMT. Lethally irradiated C3H (H-2k) mice were injected with 5 × 10^6 TCD-BM cells from either WT or H2-Ab1<sup>−/−</sup> B6 (H-2<sup>b</sup>) donors. After TCD-BMT from H2-Ab1<sup>−/−</sup> B6 donors, MHC class II molecules were expressed on the radiodestructive thymic epithelium that supports positive selection but not on the radiosensitive hematopoietic elements responsible for negative selection. A flow cytometric analysis of the thymus 4 weeks after allogeneic BMT from H2-Ab1<sup>−/−</sup> B6 donors confirmed the replacement of host thymic DCs by donor-derived DCs as previously shown; more than 98% of DCs were MHC class II negative. This analysis also demonstrated the emergence of CD4 single-positive thymocytes in both H2-Ab1<sup>−/−</sup> → C3H mice and [WT → C3H] mice (data not shown), thus confirming the preservation of the ability of the thymus to perform efficient positive selection after TCD-BMT as previously shown. An analysis of the donor cell chimerism in both H2-Ab1<sup>−/−</sup> → C3H mice and [WT → C3H] mice (data not shown), thus confirming the preservation of the ability of the thymus to perform efficient positive selection after TCD-BMT as previously shown. An analysis of the donor cell chimerism in both H2-Ab1<sup>−/−</sup> → C3H mice and [WT → C3H] mice (data not shown), thus confirming the preservation of the ability of the thymus to perform efficient positive selection after TCD-BMT as previously shown. An analysis of the donor cell chimerism in both H2-Ab1<sup>−/−</sup> → C3H mice and [WT → C3H] mice (data not shown), thus confirming the preservation of the ability of the thymus to perform efficient positive selection after TCD-BMT as previously shown.

Interestingly, the H2-Ab1<sup>−/−</sup> → C3H mice began to lose weight 6 weeks after BMT. Weight loss was significantly greater in the H2-Ab1<sup>−/−</sup> → C3H mice than in the [WT → C3H] mice at 7 weeks after BMT and thereafter (Figure 1A). This systemic illness was lethal so that only 16% survived on day 100 after BMT (Figure 1B, *P < .001). Similar results were obtained when BALB/c (H-2<sup>d</sup>) mice underwent transplantation with TCD-BM from H2-Ab1<sup>−/−</sup> B6 donors. Seventy-one percent of the [WT → C3H] mice died from GVHD by day 100 after BMT (Figure 1C).

We then determined whether the thymus played a causative role in the development of GVHD. C3H mice were thymectomized prior to BMT and then underwent transplantation with TCD-BM cells from H2-Ab1<sup>−/−</sup> B6 donors. Control, unthymectomized C3H recipients of TCD-BMT from H2-Ab1<sup>−/−</sup> B6 donors again developed severe and lethal GVHD with only a 33% survival on day 80 after BMT, whereas all thymectomized recipients survived this period (Figure 1D) without any significant weight loss (Figure 1E). We thus confirmed the causal association of the thymus with the development of this disease and ruled out the possibility that the peripheral expansion of mature T cells contaminated in TCD-BM may have caused GVHD.

As expected, a histologic examination of the skin, liver, and salivary glands 10 weeks after BMT in the [WT → C3H] mice showed no signs of GVHD (Figure 2A,C,E). In contrast, the H2-Ab1<sup>−/−</sup> → C3H mice showed standard pathologic features of chronic GVHD in the skin (Figure 2B), liver (Figure 2D), and salivary glands (Figure 2F). The skin pathology showed epidermal atrophy, follicular dropout, fat loss, and dermal fibrosis with scarce cell infiltration (Figure 2B). The liver pathology showed mononuclear cell infiltrates, bile duct loss, and fibrosis in the portal area (Figure 2D). Dry mouth is one of diagnostic features of chronic GVHD. We found lymphocytic inflammation, fibrosis, and atrophy of acinar tissue in the salivary glands (Figure 2F). In addition, we observed the interstitial and alveolar infiltrate of lymphocytes and macrophages in the lungs (data not shown). Pathology scores of the skin were significantly higher in the H2-Ab1<sup>−/−</sup> → C3H mice than in the controls (Table 1). Liver injury was also demonstrated.

Figure 1. Thymic-dependent GVHD after allogeneic TCD-BMT from MHC class II–deficient donors. C3H mice were irradiated and underwent transplantation with TCD-BM from either control WT or H2-Ab1<sup>−/−</sup> B6 donors. Weight changes as the mean ± SE (A) and survivals (B) after BMT are shown. Data from 3 similar experiments are combined: [WT → C3H], n = 22; H2-Ab1<sup>−/−</sup> → C3H, n = 27. (C) Lethally irradiated BALB/c mice underwent transplantation with TCD-BM from either control WT or H2-Ab1<sup>−/−</sup> B6 donors. The survivals after BMT are shown. [WT → BALB/c], n = 6; H2-Ab1<sup>−/−</sup> → BALB/c, n = 7. (D-E) C3H mice were thymectomized (Tx) and underwent transplantation with TCD-BM from H2-Ab1<sup>−/−</sup> B6 donors following 13 Gy TBI (N, n = 4). Nonthymectomized C3H mice also underwent transplantation with TCD-BM from WT (C, n = 4) and H2-Ab1<sup>−/−</sup> (N, n = 6) B6 donors following TBI. Survivals (D) and weight changes as the mean ± SE (E) are shown. *P < .05, **P < .001.

Figure 2. Histologic analysis of H2-Ab1<sup>−/−</sup> → C3H mice showed pathologic features similar to human chronic GVHD. The histologic findings of the skin (A-B), liver (C-D), and salivary glands (E-F) from [WT → C3H] mice and [H2-Ab1<sup>−/−</sup> → C3H] mice are shown. Sclerodermatous skin changes, such as epidermal atrophy, fat loss, follicular dropout, and dermal thickness (B); bile duct loss and fibrosis in the portal area and mild perportal mononuclear infiltrates in the liver (D, arrow); and lymphocyte inflammation, fibrosis, and atrophy of acinar tissue in the salivary glands (F, arrow) were observed in H2-Ab1<sup>−/−</sup> → C3H mice (original magnification, ×100).
The skin was analyzed using the histopathologic scoring system described in "Assessment of GVHD" 10 weeks after BMT. For [H2-Ab1−/− → C3H] mice, \( n = 16 \). The data are expressed as the mean ± SD. Scores were 0.0 ± 0.0 for all 15 [WT → C3H] mice.

\[ P < .01. \]

by the elevation of the serum alanine transferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin levels at 10 weeks after BMT (Table 2). [H2-Ab1−/− → C3H] mice also developed pancytopenia at 10 weeks after BMT (Table 2). Similar pathologic changes were also observed in the [H2-Ab1−/− → BALB/c] (data not shown).

To examine whether CD8+ cells are involved in the effector mechanisms, [H2-Ab1−/− → C3H] chimeras were depleted of CD8+ cells after BMT by chronic administration of anti-CD8 mAb. Flow cytometric analysis of the spleen 6 weeks after BMT confirmed the effective elimination of CD8+ cells (less than 0.2%). However, GVHD did develop in CD8-depleted chimeras as severe as control [H2-Ab1−/− → C3H] chimeras (data not shown), suggesting that CD4+ cells alone are sufficient for the development of GVHD.

Adoptive transfer of the pathogenic CD4+ T cells caused acute GVHD in B6 recipients

To determine the emergence of donor- or host-reactive CD4+ T cells in these mice, CD4+ T cells were isolated from spleens 6 weeks after BMT. These cells were stimulated with irradiated DCs either from B6 or C3H mice to determine the proliferative responses. As expected, CD4+ T cells isolated from [WT → C3H] mice did not respond to B6 (donor) or C3H (recipient) stimulators (Figure 3A). In contrast, [H2-Ab1−/− → C3H] CD4+ T cells proliferated vigorously in response to B6 stimulators. Similarly, [H2-Ab1−/− → BALB/c] CD4+ T cells were also B6 reactive (Figure 3B). Cell division of CFSE-labeled donor T cells was also analyzed in culture. [H2-Ab1−/− → C3H] CD4+ T cells divided in response to B6 stimulators and CD3 stimulation but not to C3H stimulators (Figure 3C). Thus, these results demonstrate the emergence of primarily donor-reactive CD4+ T cells.

[H2-Ab1−/− → C3H] chimeras lack MHC class II expression on antigen-presenting cells (APCs) in the periphery and thus are not relevant to clinical BMT. Also, it is not clear whether GVHD in these mice is due to the impaired thymic negative selection, due to the lack of MHC class II in the periphery, or both. We therefore tested whether [H2-Ab1−/− → C3H] CD4+ T cells could cause GVHD after adoptive transfer to B6 or C3H mice. A total of 1 × 107 [WT → C3H] or [H2-Ab1−/− → C3H] CD4+ T cells were transferred to lethally irradiated B6 or C3H mice together with host-type TCD-BM. In the control, the transfer of [WT → C3H] CD4+ T cells did not cause GVHD in B6 or C3H mice, as expected (Figure 3D-E). In contrast, the transfer of [H2-Ab1−/− → C3H] CD4+ T cells caused severe and lethal disease in B6 recipients (Figure 3D-E). A pathologic analysis of the liver and intestine showed standard pathologic features of acute GVHD. In the intestine villous atrophy, crypt cell apoptosis, and lymphocytic infiltrates were noted (Figure 3F, left panel). In the liver, mononuclear cells densely infiltrated the bile duct epithelium and the portal area with necrotic hepatocytes (Figure 3F, right panel). These mice did not meet the criteria for chronic GVHD (Table 3). In contrast, the transfer of [H2-Ab1−/− → C3H] CD4+ T cells did not cause GVHD in C3H recipients (Figure 3D-E). Thus, both in vitro and in vivo experiments demonstrated that pathogenic CD4+ T cells that developed in [H2-Ab1−/− → C3H] mice were primarily B6 reactive.

Adoptive transfer of the pathogenic CD4+ T cells caused chronic GVHD in C3H recipients in the presence of B6 APCs

We then investigated whether these pathogenic T cells that escaped from thymic negative selection could cause GVHD in C3H mice in the presence of B6-derived APCs. CD4+ T cells isolated from the [H2-Ab1−/− → C3H] mice at 6 weeks after transplantation were transferred to lethally irradiated C3H mice together with either B6 TCD-BM or C3H TCD-BM. Again, the transfer of [H2-Ab1−/− → C3H] CD4+ T cells did not cause GVHD in C3H recipients when transferred with C3H TCD-BM (Figure 4A). Interestingly, these cells transmitted lethal GVHD in secondary C3H recipients when transferred with B6 TCD-BM. Recipients of [H2-Ab1−/− → C3H] CD4+ T cells and B6 TCD-BM began to lose weight 4 weeks after transfer. Weight loss was significantly greater in these mice than in the recipients of [H2-Ab1−/− → C3H] CD4+ T cells and C3H TCD-BM at 6 weeks after transfer and thereafter (Figure 4B). Recipients of [H2-Ab1−/− → C3H] CD4+ T cells and C3H TCD-BM looked healthy (Figure 4C, left panel), but those of [H2-Ab1−/− → C3H] cells and B6 TCD-BM were hunched and displayed scleroderma skin changes (Figure 4C, right panel). This systemic illness was lethal so that only 16% survived on day 80 after transfer (Figure 4A, \( P < .001 \)).

A histologic examination of the skin, liver, and salivary glands 6 weeks after transfer of the [WT → C3H] CD4+ T cells and B6 TCD-BM showed no signs of GVHD (Figure 5A,C,E). In contrast, the transfer of [H2-Ab1−/− → C3H] CD4+ T cells and B6 TCD-BM produced similar chronic GVHD to the primary [H2-Ab1−/− → C3H] in the skin, liver, and salivary glands (Figure 5B,D,F). In these recipients, the skin pathology scores were significantly elevated (Figure 4D). Furthermore, these mice showed profound

### Table 1. Development of skin GVHD in [H2-Ab1−/− → C3H] mice

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Score</th>
<th>( n = 16 )</th>
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<tbody>
<tr>
<td>Thickened dermis</td>
<td>1.6 ± 0.5*</td>
<td>16</td>
</tr>
<tr>
<td>Fat loss</td>
<td>1.9 ± 0.2*</td>
<td>16</td>
</tr>
<tr>
<td>Epidermis atrophy</td>
<td>0.8 ± 0.3*</td>
<td>16</td>
</tr>
<tr>
<td>Follicle loss</td>
<td>0.9 ± 0.5*</td>
<td>16</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0.0 ± 0.0</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>5.3 ± 1.0*</td>
<td>16</td>
</tr>
</tbody>
</table>

The skin was analyzed using the histopathologic scoring system described in "Assessment of GVHD" 10 weeks after BMT. For [H2-Ab1−/− → C3H] mice, \( n = 16 \). The data are expressed as the mean ± SD. Scores were 0.0 ± 0.0 for all 15 [WT → C3H] mice.

\[ P < .01. \]

### Table 2. Development of liver injury and pancytopenia in the [H2-Ab1−/− → C3H] mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Naive [WT → C3H]</th>
<th>[H2-Ab1−/− → C3H]</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST level, IU/L</td>
<td>72.2 ± 11.3</td>
<td>94.3 ± 26.9</td>
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<tr>
<td>ALT level, IU/L</td>
<td>27.0 ± 5.4</td>
<td>43.3 ± 15.8</td>
</tr>
<tr>
<td>ALP level, IU/L</td>
<td>312.0 ± 126.0</td>
<td>446.5 ± 22.7</td>
</tr>
<tr>
<td>Bilirubin, mg/dL</td>
<td>0.08 ± 0.03</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>CBCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cell count, ( \times 10^{11} /L )</td>
<td>10.6 ± 3.8</td>
<td>13.0 ± 3.1</td>
</tr>
<tr>
<td>Red blood cell count, ( \times 10^{12} /L )</td>
<td>8.9 ± 0.3</td>
<td>7.9 ± 8.3</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>132.0 ± 5.0</td>
<td>129.6 ± 14.2</td>
</tr>
<tr>
<td>Platelet count, ( \times 10^{12} /L )</td>
<td>731.3 ± 83.0</td>
<td>798.0 ± 17.7</td>
</tr>
</tbody>
</table>

The complete blood counts (CBCs) and serum indices of liver damage were measured 10 weeks after BMT. The data are expressed as the mean ± SD.

To convert bilirubin level from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 17.1.

† \( P < .05 \), [WT → C3H] versus [H2-Ab1−/− → C3H].

‡ \( P < .01 \), [WT → C3H] versus [H2-Ab1−/− → C3H].
leukocytopenia and thrombocytopenia at 7 weeks after transfer (Figure 4E). These mice did not meet the criteria for acute GVHD (Table 3). These results suggest that \( [H2-Ab1^{+/+} \rightarrow C3H] \) CD4+ T cells cause acute GVHD when target epithelium expresses B6 antigens but chronic GVHD when B6 antigens are provided by hematopoietic cells in the absence of B6 antigen expression on target epithelium.

To further confirm the requirement of B6-derived APCs for the disease, we created \( [B6 \rightarrow C3H] \) and \( [C3H \rightarrow C3H] \) chimeras by reconstituting lethally irradiated C3H mice with TCD-BM from B6 and C3H mice, respectively. Four months later, a flow cytometric analysis of splenic DCs isolated from these animals showed complete replacement by donor-derived DCs (data not shown) as previously described.\(^{37}\) Next, these chimeric mice were sublethally irradiated and injected with \( 1 \times 10^7 \) CD4+ T cells isolated either from the \( [WT \rightarrow C3H] \) or \( [H2-Ab1^{-/-} \rightarrow C3H] \) mice alone without TCD-BM. In the control, the transfer of the \( [WT \rightarrow C3H] \) CD4+ T cells did not cause GVHD in either the \( [B6 \rightarrow C3H] \) or \( [C3H \rightarrow C3H] \) chimeras (Figure 6). However, the transfer of \( [H2-Ab1^{-/-} \rightarrow C3H] \) CD4+ T cells did cause a significant weight loss and lethal GVHD in the \( [B6 \rightarrow C3H] \) chimeras but not in the \( [C3H \rightarrow C3H] \) chimeras, thus confirming the requirement of B6-derived APCs for the transmission of the disease to secondary recipients.

We finally evaluated whether the impaired MHC class II–mediated thymic negative selection affects development of CD4+ CD25+ regulatory T (Treg) cells. Cells were isolated from the

<table>
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<th>Table 3. Number of mice that fall into either category of acute or chronic GVHD</th>
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<tr>
<td>( [H2-Ab1^{+/+} \rightarrow C3H] ) CD4+ T cells transferred to lethally irradiated B6 or C3H mice together with TCD-BM from B6 mice.</td>
</tr>
<tr>
<td>( [H2-Ab1^{-/-} \rightarrow C3H] ) CD4+ T cells transferred to lethally irradiated C3H mice together with TCD-BM from B6 mice.</td>
</tr>
<tr>
<td>Acute GVHD, no. of mice</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

A total of \( 1 \times 10^7 \) \( [H2-Ab1^{+/+} \rightarrow C3H] \) CD4+ T cells were transferred to lethally irradiated B6 or C3H mice together with TCD-BM from B6 mice.
spleen and thymus 5 and 8 weeks after BMT. A flow cytometric analysis showed that the numbers of CD4+ Foxp3+ T cells in the spleen and thymus were comparable between [WT → C3H] and [H2-Ab1−/− → C3H] mice (Figure 7A-B). To examine whether these Treg cells are functional in [H2-Ab1−/− → C3H] mice, these mice were depleted of CD25+ cells after TCD-BMT by chronic administration of anti-CD25 mAb. GVHD was more severe in CD25-depleted mice than in controls (Figure 7C), suggesting that Treg cells are functional in these mice but not sufficient to inhibit GVHD.

Discussion

Thymic-dependent GVHD in our model is similar to human chronic GVHD in terms of its clinical manifestations and pathology. Multiple organs are involved, such as the skin, liver, lung, salivary glands, and hematopoietic system. In particular, skin sclerodermatous change and destruction of salivary glands are frequent and specific manifestations of chronic GVHD in humans. Hepatic disease is characterized by bile duct mononuclear cell infiltrates followed by bile duct loss and fibrosis. Such a “vanishing bile duct” is also one of the key histologic features of hepatic chronic GVHD. Other features include lung injury, immunodeficiency, and pancytopenia; these are also consistent with human chronic GVHD. Thymopoiesis declines with age; however, it is now clear that a substantial output is maintained late into adulthood following HSCT as demonstrated by the observation that the number of naive or T-cell–receptor excision circle–containing (TREC+) CD4+ T cells usually increases between 3 and 12 months after grafting. In mice, the thymic-dependent CD4+ T cells significantly repopulate recipients by 6 weeks after transplantation.

Experimental studies and clinical observations have convincingly shown that GVHD is initiated by donor T cells. The donor T-cell repopulation after HSCT results from both the peripheral expansion of mature donor T cells and the thymic-dependent generation of donor T cells from donor hematopoietic stem cells. It has been shown that the former pathway plays an important role in both acute and chronic GVHD in murine models. Thymectomy of the recipients prior to BMT prevented the development of disease similar to human chronic GVHD after allogeneic BMT. Taken in that context, our data would suggest that both the peripheral expansion of mature donor T cells and thymic-dependent generation of donor T cells from donor hematopoietic stem cells may play a role in the induction of chronic GVHD.

A disrupted peripheral regulatory mechanism is also responsible for the development of GVHD as well as autoimmune diseases. It is thus possible that dysfunction of peripheral regulatory mechanisms may be involved in this disease process. However, we found that the numbers of CD4+ CD25+ Foxp3+ Treg cells in [H2-Ab1−/− → C3H] mice were equivalent to those in controls, and these cells were functional in these mice. Thus, functional Treg cells normally developed in these mice, as they did in mice in which the MHC class II expression was limited on thymic epithelial cells but not on the hematopoietically derived element. Also, it has been shown that MHC class II–deficient Treg
cells are as suppressive as MHC class II–bearing Treg cells. Nonetheless, the role of non-CD4+CD25+ Foxp3+ Treg cells will be explored in the future.

In vitro experiments and adoptive transfer experiments suggest that [H2-Ab1−/− → C3H] CD4+ T cells are reactive to self-antigens. The transfer of these CD4+ T cells caused GVHD in B6 recipients but not in C3H recipients. Thus, there seems to be some degree of partial tolerization to C3H antigens. Negative selection can be also mediated by thymic medullary epithelial cells (MECs)52 and, thus, C3H-derived MECs may be sufficient to eliminate most C3H-reactive T cells. Nonetheless, these cells can transmit GVHD to C3H mice when a repopulation of B6-derived hematopoiesis has occurred. Stimulation of B6-reactive T cells with B6 antigens may enhance the ability of these cells to perpetuate the disease. Similar to our study, several previous experiments have shown that stimulation of B6-reactive T cells with B6 antigens may enhance the ability of these cells, but depletion of CD8+ T cells did not abrogate GVHD in [H2-Ab1−/− → BALB/c] BMT model of chronic GVHD. Tivol and colleagues54 showed that chronic GVHD in a B6 → BALB/c BMT model results in the emergence of B6-reactive donor T cells that can cause severe autoimmune colitis in B6 recipients but not in BALB/c recipients after transfer. Similar to our findings, transfer of the GvHD T cells could induce severe colitis in BALB/c recipients only when B6-derived APCs were present.54 It is possible that impaired negative selection in these animals might have occurred due to destruction of donor-derived APCs in the thymus. However, the role of thymus was not addressed in this B6 → BALB/c model. Conditioning damages the thymus and donor T cells invade the thymus, and the thymic structure is destroyed. Therefore, both donor- and host-reactive T cells could be generated.

Given the requirement of B6 APCs for the transmission of chronic GVHD to C3H mice, it is puzzling that chronic GVHD developed in [H2-Ab1−/− → C3H] chimeras in the absence of MHC class II expression on B6-derived APCs. We considered the involvement of CD8+ T cells stimulated by abnormal CD4+ T cells, but depletion of CD8+ T cells did not abrogate GVHD in [H2-Ab1−/− → C3H] chimeras. MHC class II expression on APCs may influence the reactivity of postthymic T cells, referred to as “tuning.”55 Mature CD4+ T cells become hyperreactive against both syngeneic and allogeneic skin grafts upon transfer into MHC class II–deficient hosts.55 Therefore, it is tempting to speculate that absence of MHC class II expression on APCs in [H2-Ab1−/− → C3H] chimeras dynamically reduces the activation threshold of CD4+ T cells and caused chronic GVHD in these mice. In transfer experiments, MHC class II–positive B6-derived APCs directly stimulate B6-reactive CD4+ T cells to cause GVHD, whereas C3H-derived APCs may “tune” the pathogenic CD4+ T cells in the absence of B6-derived APCs.

The transfer of [H2-Ab1−/− → C3H] CD4+ T cells caused acute, not chronic, GVHD in B6 mice. This result is consistent with our previous observations that the transfer of CD4+ T cells from [H2-Ab1−/− → B6] mice caused acute GVHD in syngeneic B6 mice.52 Notably, however, these are not relevant to clinical BMT, because there is no clinical situation in which donor T cells infuse back to donors. On the other hand, it is intriguing that [H2-Ab1−/− → C3H] and [H2-Ab1−/− → BALB/c] CD4+ T cells caused chronic GVHD in allogeneic C3H and BALB/c mice, respectively, after transfer with B6 TCD-BM in the current study. Although the precise mechanisms of how these cells differentially cause acute and chronic GVHD remain to be elucidated, expression of B6 antigens on target epithelium may cause a more inflammatory form of GVHD, acute GVHD, through the direct attack on target epithelial cells by B6-reactive T cells, whereas absence of B6 antigens on target epithelium may cause less inflammatory chronic GVHD in the presence of B6-derived APCs. In this scenario, it is possible that B6-reactive T cells attack the target epithelium in C3H recipients through the secretion of fibrosing and inflammatory cytokines such as transforming growth factor-β,64,65 promotion of B-cell activation and autoantibody production,2,16,60 or nonspecific bystander lysis.62

We have shown that alloreactive donor T cells could damage epithelial cells that lack alloantigen expression when alloantigens are expressed on APCs in MHC-mismatched mouse models of BMT.37 In these experiments, alloantigen expression on APCs is sufficient for the migration of donor T cells into target tissues.37 Thus, APCs that reside in GVHD target tissues may recruit and stimulate alloreactive donor T cells to cause bystander injury of the surrounding epithelial cells.37,62 Alternately, it is still possible that B6-reactive CD4+ T cells cross-react on C3H or BALB/c sufficiently to cause chronic GVHD, although we are not able to demonstrate such cross-reactivity in vitro and adoptive transfer experiments. There are now accumulating evidences of cross-reactivity between self-antigens and alloantigens. In K14 mice expressing MHC class II only on thymic cortical epithelium, negative selection was impaired and autoreactive CD4+ T cells generated.63 Surprisingly, more than half of the T-cell hybrids established from the K14 CD4+ T cells reacted to both B6-derived APCs and allogeneic APCs.64 Thus, stimulation of B6-reactive T cells with B6 antigens may enhance reactivity to C3H antigens sufficiently to cause GVHD. Thus, the reactivity and/or frequency of host-reactive T cells may determine the development of acute or chronic GVHD.

![Figure 7. The numbers of CD4+ Foxp3+ T cells were comparable between WT → C3H and H2-Ab1−/− → C3H mice. Cells isolated from the thymus and spleen from WT → C3H and H2-Ab1−/− → C3H mice were stained for CD4 and Foxp3 mAbs. (A) The dot plots show the 2-color staining pattern for CD4-FITC and Foxp3-PE on CD4 single-positive thymocytes and splenocytes 5 weeks after BMT. (B) The numbers of CD4+ Foxp3+ T cells on the CD4 single-positive thymocytes and splenocytes from [WT → C3H] or [H2-Ab1−/− → C3H] mice were enumerated 5 and 8 weeks after BMT (n = 3 per group). (C) C3H mice were irradiated and underwent transplantation with TCD-BM from either control WT or H2-Ab1−/− B6 donors. After BMT, mice were injected with anti-CD25 mAbs or control Abs. Weight changes as the mean ± SE after BMT are shown. ○, [WT → C3H], n = 4; ●, control [H2-Ab1−/− → C3H], n = 6; □, [H2-Ab1−/− → C3H] treated with anti-CD25 mAbs, n = 6. *P < .05.](image-url)
It remains to be investigated whether chronic GVHD is a later manifestation of acute GVHD or has a different pathogenesis involving different effector cells. Both could be true, but previous studies\textsuperscript{16,34,60} and the current study favor the latter hypothesis. Several attempts to decrease acute GVHD does not result in a reduction of the chronic GVHD rates.\textsuperscript{5-10} Current study suggests that the pathogenesis of acute GVHD and chronic GVHD is different, and our model will be useful to study the pathogenesis and pathophysiology of chronic GVHD. Our results also suggest that an improvement in the thymic function may have a potential to decrease chronic GVHD.

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