in pediatric steroid-refractory aGVHD in Canada and New Zealand as a cell-based medicine [24].

We confirmed that third party-derived bone marrow MSCs are safe and effective for patients with steroid-resistant aGVHD. In our study, only MSCs were given to patients with steroid-refractory GVHD as soon as possible after the diagnosis of steroid-refractory GVHD. Since the cell dose of infused MSCs was constant and the number of MSC infusions was strictly scheduled, our results are reliable to estimate the effects of MSCs on steroid-resistant aGVHD. A high CR rate and good OS were obtained. Of note, gut aGVHD comprised 71 % (10 of 14 patients) of our patients and all the patients except one showed CR. These results are consistent with others, i.e., MSCs have a favorable clinical effect on gut aGVHD [7, 12, 23]. Our trials did not include patients with grade IV aGVHD. Therefore, the good results in our studies may have been overestimated. As in other reports, no apparent adverse effects associated with MSC therapy were observed in short-term and long-term observations.

The presence of fetal bovine serum is necessary for standard conditions for MSC expansion [25]. However, it is better not to use animal products to avoid unknown infections and other complications. von Bonin et al. and Lucchini et al. showed the usefulness of platelet-lysate-expanded bone marrow MSCs for steroid-refractory aGVHD [13, 15]. Alternatively, MSC donor serum can be used for MSC expansion. Arima et al. and Pérez-Simon et al. successfully treated steroid-refractory aGVHD with bone marrow MSCs expanded in a medium supplemented with autologous serum [16, 18]. It is not known which MSC culture is the best in terms of the safety and growth of MSCs. Ideally, a serum-free culture of MSCs should be introduced in a clinical setting [26].

After the completion of the JCR-031-201 and JCR-031-202 trials, we started a phase III trial using JR-031 focusing on steroid-refractory grade III or IV aGVHD. In the near future, the results of this study will be published.

Conflict of interest K. Muroi and K. Ozawa received payment for consultancy from JCR Pharmaceuticals Co., Ltd. Other authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Frequency of $CD4^+FOXP3^+$ regulatory T-cells at early stages after HLA-mismatched allogeneic hematopoietic SCT predicts the incidence of acute GVHD

T Fujioka, H Tamaki, K Ikegame, S Yoshihara, K Taniguchi, K Kaida, R Kato, T Inoue, J Nakata, S Ishii, T Soma, M Okada and H Ogawa

Acute GVHD (aGVHD) is a major obstacle to allogeneic hematopoietic SCT (alloHSCT). Although it is thought that aGVHD is initiated in secondary lymphoid organs at a very early stage of alloHSCT, whether $CD4^+FOXP3^+$ regulatory T-cells (Tregs) have an impact on aGVHD development during this period remains unclear. Here, we measured Tregs in peripheral blood as early as possible after HLA-mismatched alloHSCT, and assessed the incidence of aGVHD. Flow cytometric analyses revealed that at the second week after HSCT, patients with aGVHD had significantly ($P=0.018$) lower Treg:$CD4^+$T-cell ratios than those without aGVHD. As these differences were seen before the development of aGVHD, these ratios can predict the incidence of aGVHD. The cumulative incidence of aGVHD in patients with ratios of $<9\%$ was significantly higher than that in patients with ratios of $\geqslant 9\%$ ($P=0.0082$, log-rank test). Additionally, the specific ratio of Tregs:$CD4^+$T-cells was the most significant value among all other possible lymphocyte-associated ratios and absolute cell counts. These findings suggest that the ratio of Tregs:$CD4^+$T-cells at the second week post HLA-mismatched alloHSCT might be a potent predictor of aGVHD in these patients. The practical efficacy of this finding should be verified in further interventional studies.

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Keywords: regulatory T-cells; GVHD; allogeneic SCT; HLA mismatch

INTRODUCTION

Although allogeneic hematopoietic SCT (alloHSCT) has the potential to cure many hematological disorders, GVHD continues to be a major obstacle associated with morbidity and mortality. Naturally occurring regulatory T-cells (Tregs) initially found in $CD4^+CD25^{high}$T-cell fractions[1,2] suppress autoreactive[1] and alloreactive[3–5] immunoreactions. Other researchers have investigated the relationship between the frequency of $CD3^+CD4^+CD25^{high}$Tregs in peripheral blood and the incidence of GVHD after alloHSCT, but results have been inconsistent, possibly due to differences in the definition of $CD25^{high}$.[6–8] The intracellular protein derived from the *FOXP3* gene has since been detected using flow cytometry, and is recognized as both a master regulatory gene and a unique marker for these Tregs.[9,10] This procedure enables the specific measurement of Tregs, and distinguishes them from activated conventional $CD4^+CD25^+$ T-cells. Subsequent studies have applied this procedure and suggested the role of Tregs in attenuating GVHD, mostly after HLA-matched alloHSCT.[11–14]

Here we examined Treg frequencies in the peripheral blood of patients who received alloHSCT from an HLA-mismatched related donor without T-cell depletion. As donor T-cells rapidly recover under our HSCT clinical protocol,[15,16] we analyzed the frequencies of Tregs and other lymphocyte populations as early as possible following HSCT, and examined the relationship between Treg frequency and the subsequent incidence of acute GVHD (aGVHD).

PATIENTS AND METHODS

Patients and samples

Forty-seven patients who underwent alloHSCT from partially HLA-mismatched related donors without T-cell depletion were evaluated. All patients received treatment at the Hyogo College of Medicine Hospital (Nishinomiya City, Japan) between July 2007 and August 2010 in accordance with the protocols approved by the institutional review board. Of these 47 patients, 45 received HLA-haploidentical HSCT. Patient characteristics are summarized in Table 1. After the provision of written informed consent, peripheral blood samples were obtained weekly on a fixed day of the week from the first to the eighth week after transplantation. Data acquired between day 1 and 7 were accordingly defined as data of the first week, those between day 8 and 14 as data of the second week, and so on.

Transplant procedure

Thirty and seventeen patients were preconditioned with a nonmyeloablative and myeloablative regimen, respectively, as reported previously.[15,16] In brief, the nonmyeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, for 6 days), BU (3.2 mg/kg/day, for 2 days, i.v.), and either anti-T-lymphocyte globulin (Fresenius Biotech GmbH, Munich, Germany) or anti-thymocyte globulin (Genzyme, Cambridge, MA, USA) (8 mg/kg or 2–4 mg/kg of the total dose, respectively). The myeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, 4 days), cytosine arabinoside (2 g/m^2/day, 4 times over 2 days), CY (60 mg/kg/day, for 2 days) and TBI (8 Gy delivered in 4 fractions). The GVHD prophylaxis regimen for nonmyeloablative HSCT consisted of tacrolimus (0.02 mg/kg/day) and methylprednisolone (1 mg/kg/day), and that for myeloablative HSCT consisted of tacrolimus (0.03 mg/kg/day), MTX

Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan. Correspondence: Dr T Fujioka, Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya-city, Hyogo 663-8501, Japan.
E-mail: fujioka@hyo-med.ac.jp
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Table 1. Patient characteristics

	No GVHD	*GVHD*	P-*value*
Number	25	22	
Median age	37	34	0.49
Sex			*0.33*
Male	9	11	
Female	16	11	
Diagnosis			*0.91*
ALL	6	8	
AML	6	6	
Non-Hodgkin lymphoma	7	4	
Myelodysplastic syndrome	3	2	
Hodgkin lymphoma	1	1	
CLL	1	1	
CML	1	0	
Conditioning intensity			*0.98*
Nonmyeloablative	16	14	
Myeloablative	9	8	
Source of stem cells			*0.16*
PBSC	12	15	
BM	13	7	
GVHD grade			*NA*
I		11	
II		7	
III		4	
IV		0	

Abbreviation: NA = not applicable.

(10 mg/m^2 on day 1 and 7 mg/m^2 on day 3), methylprednisolone (2 mg/kg/day) and mycophenolate mofetil (15 mg/kg). After transplantation, degrees of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood were assessed by quantitative PCR for STR markers, as previously reported.[17] Assessment of aGVHD was based on clinical symptoms in accordance with commonly accepted criteria.[18,19] Unless patient condition precluded them, skin, liver and gastrointestinal tract biopsies were performed to support the diagnoses. Gastric biopsy was essentially required for the diagnosis of gut GVHD without manifest diarrhea (stage 1).

Flow cytometric analysis of Treg

Peripheral blood samples were collected using EDTA anticoagulant, and PBMCs were isolated by density-gradient centrifugation for analysis without cryopreservation. Flow cytometric analysis was performed using a Coulter cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) with CXP software (Beckman Coulter), using the following Abs: FITC-conjugated anti-CD3, phycoerythrin-Texas Red energy-coupled dye-conjugated anti-CD25, and phycoerythrin-Cy5-conjugated anti-CD4 (Beckman Coulter). For FOXP3 intracellular staining, the phycoerythrin-conjugated anti-FOXP3 Staining Set (eBioscience, San Diego, CA, USA) was used according to the manufacturer's instructions. FOXP3 staining was performed independently after staining with other Abs.

Statistical analysis

Differences in characteristics between patient groups were assessed by the Mann – Whitney *U*-test for continuous variables and the χ^2 test for categorical values. Median Treg frequencies were compared using the Mann – Whitney *U*-test. Treg frequencies were adjusted for differences between patients with and without aGVHD by multiple regression with logistic analysis. The sensitivity and specificity of Tregs in predicting aGVHD were assessed by receiver operating characteristic curve analysis. Cumulative incidences of aGVHD were plotted according to the Kaplan – Meier method and compared using the log-rank test.

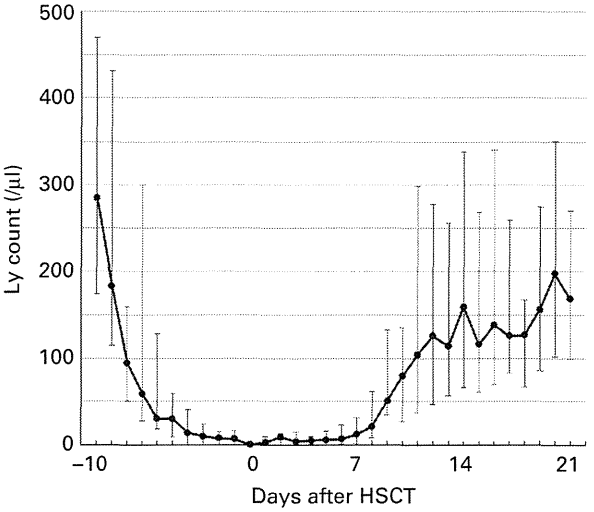

Figure 1. Recovery of lymphocyte (Ly) counts after HLA-mismatched HSCT. Median lymphocyte counts in peripheral blood after HLA-mismatched HSCT are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively.

RESULTS

Patients

Of the 47 patients, 22 presented with aGVHD *vs* 25 who did not (Table 1). The onset of aGVHD occurred at a median of 38 days after transplantation (range: 14–102). None of the characteristics examined had any significant impact on aGVHD incidence. As described previously, the degree of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood achieves the complete donor type within 2 weeks after HLA-mismatched HSCT in our hospital.[15–17] Here, assessment once per week confirmed that on average complete donor-type chimerism in T-cells was achieved on day 10 (median, range: 5–23). Lymphocytes recovered during the second week (Figure 1) were therefore considered to consist almost entirely of donor-originated lymphocytes.

Flow cytometric analysis of Tregs

Representative results of a patient 4 weeks after HSCT are shown in Figures 2a–d. FOXP3$^+$ Tregs were analyzed using a flow cytometric plot gated by CD3$^+$CD4$^+$ fractions (Figure 2a). Although CD25 staining alone showed a large overlap between CD25$^+$ and CD25$^-$ cells (Figure 2c), FOXP3 staining was able to separate FOXP3$^+$ cells as an isolated population (Figure 2d). As demonstrated previously, CD25 staining alone is frequently incapable of revealing an unequivocal boundary that discriminates Tregs from CD4$^+$CD25$^+$-activated conventional T-cells in almost all cases.[20] As FOXP3 staining has apparent objectivity and, moreover, FOXP3 is the key molecule for this type of Treg,[9,10] we defined Tregs simply as CD4$^+$FOXP3$^+$T-cells, regardless of CD25 expression.

Treg:CD4$^+$T-cell ratios at the second week were significantly lower in patients with aGVHD

As lymphocyte numbers were markedly low during the first week, as shown in Figure 1, flow cytometric analysis was unable to detect any Tregs. By the second week after HSCT, in contrast, lymphocyte numbers increased to levels that made analysis possible in almost all cases (41 of 47 patients). Representative results of patients without and with aGVHD are shown in Figures 2e and f, respectively. Of the remaining six patients with slower lymphocyte recovery, two could be assessed at the third week,

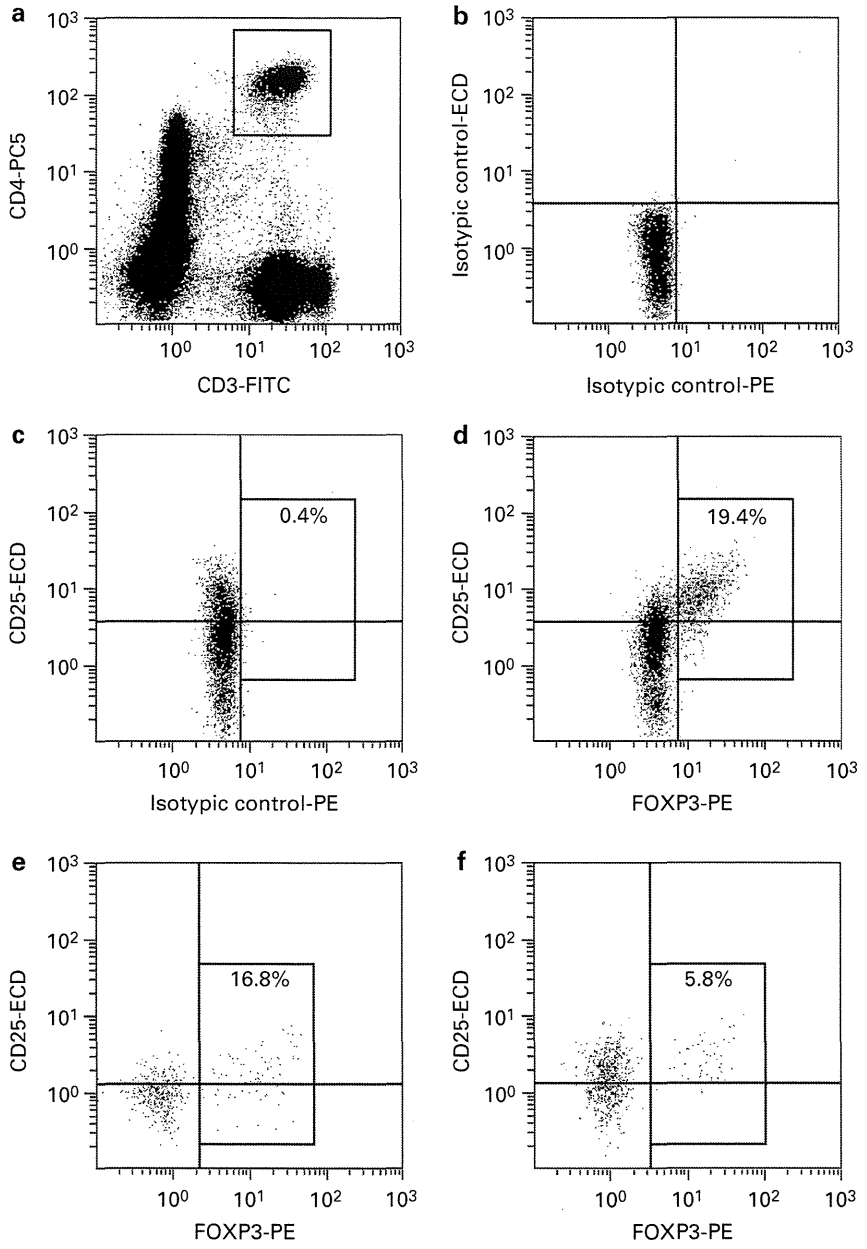

Figure 2. Flow cytometric analysis of Tregs. Representative results of four-color flow cytometric analyses performed for Tregs. Peripheral mononuclear blood cells were stained with CD3-FITC, CD4-PC5, CD25-energy-coupled dye (ECD) and FOXP3-phycoerythrin (PE). All dot plots were gated into lymphocyte populations according to forward- and side-scatter properties, and the gate of $CD3^+CD4^+$ fractions shown on plot **a** was used for the other dot plots with CD25/FOXP3 axes. The percentage of $FOXP3^+$ cells was calculated by subtracting the background percentage of the gate found in plot **c** from the gate shown on plot **d**. Plots **a**–**d** are representative results of a patient 4 weeks after HSCT. Plot **e** is a representative result of a patient without aGVHD in the second week and plot **f** is one of a patient with aGVHD in the second week.

one at the fifth week, one at the sixth week and two at the seventh week.

Figure 3 shows Treg:$CD4^+$T-cell ratios after HSCT ($n=41$), which are the most meaningful values as described in the following paragraph. On average, Tregs were collected on day 12 (median, range: 8–14) during the second week. Patients with aGVHD had significantly lower ratios in the second week after HSCT than those without aGVHD (median (range), 5.23 (0.32–44.8) *vs* 15.5 (0.00–37.1); $P=0.018$). Similar tendencies were seen during the following weeks, but the differences were not statistically significant. Multivariate analysis using logistic regression, which incorporated patient characteristics and transplantation settings, showed that Treg:$CD4^+$T-cell ratio was a unique independent and significant factor related to the incidence of aGVHD (Table 2).

Treg:$CD4^+$T-cell ratio is the most significant value among all other ratios and absolute counts

We also examined the significance of all other ratios between two major lymphocyte populations during the second week. As summarized in Table 3A, although $CD4^+$T-cell:whole T-cell ratio (median (range), 0.32 (0.09–0.78) in the aGVHD (+) group *vs* 0.16 (0.02–0.79) in the aGVHD (−) group; $P=0.026$) and

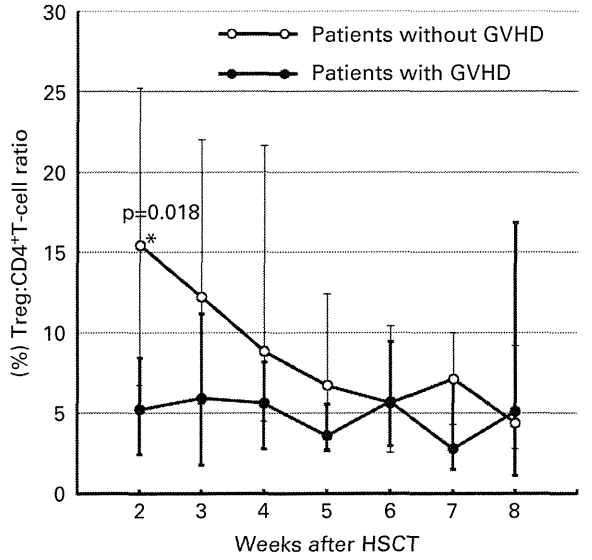

Figure 3. Frequency of Tregs in peripheral blood after HLA-mismatched HSCT. The frequencies of Tregs were assessed by flow cytometry weekly until the eighth week after HSCT. The median Treg:CD4$^+$T-cell ratios of patients with or without aGVHD are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively. Patients with aGVHD had significantly ($P=0.018$) lower median ratios at the second week after HSCT than those without aGVHD.

Table 2. Multivariate analysis

Parameter	P-*value*
Age	0.643
Sex	0.295
Diagnosis	0.774
Conditioning intensity	0.732
Source of stem cells	0.397
Treg:CD4$^+$T-cell ratio	0.032*

Abbreviation: Tregs = regulatory T-cells. *Indicates statistical significance ($P<0.05$).

CD8$^+$T-cell:CD4$^+$T-cell ratio (median (range), 1.98 (0.43–9.79) in the aGVHD (+) group *vs* 4.13 (0.38–32.3) in the aGVHD (−) group; $P=0.043$) were significant, the Treg:CD4$^+$T-cell ratio ($P=0.018$) had statistically the most significant value. Additionally, neither absolute numbers of whole lymphocytes nor the respective lymphocyte fraction (including Tregs) significantly correlated with the incidence of aGVHD (Table 3B).

Treg:CD4$^+$T-cell ratio at the second week predicts the incidence of aGVHD

As aGVHD occurred at a median of 38 days after HSCT (range, 14–102), while the significant decreases in Treg:CD4$^+$T-cell ratio were observed during the second week, this ratio can serve to predict the incidence of aGVHD. A receiver operating characteristic curve was generated by plotting the true positive rate of aGVHD against the false-positive rate for different cutoff-ratio values (Figure 4). The area under the curve was 0.73, indicating that the Treg:CD4$^+$T-cell ratio at the second week is a good predictor of aGVHD. Further analysis revealed that a cutoff-ratio value of 9% yielded the most accurate predictions of future aGVHD incidence (Figures 4, 69.6% sensitivity and 77.8% specificity). Treg:CD4$^+$T-cell ratios of $<9\%$ predicted a significantly higher incidence of aGVHD than ratios of $\geqslant 9\%$ (Figure 5, $P=0.0082$, log-rank test).

Table 3. *P*-values of each ratio (A) and absolute number (B)

A

numerator / denominator	Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
Ly		0.636	0.093	0.674	0.203	0.478
T			0.026*	0.237	0.318	0.478
CD4$^+$T				0.043*	0.774	0.018*
CD8$^+$T					0.213	0.713
B						0.139
Treg						

B

Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
0.674	0.636	0.083	0.979	0.213	0.875

*Indicates statistical significance ($P<0.05$).

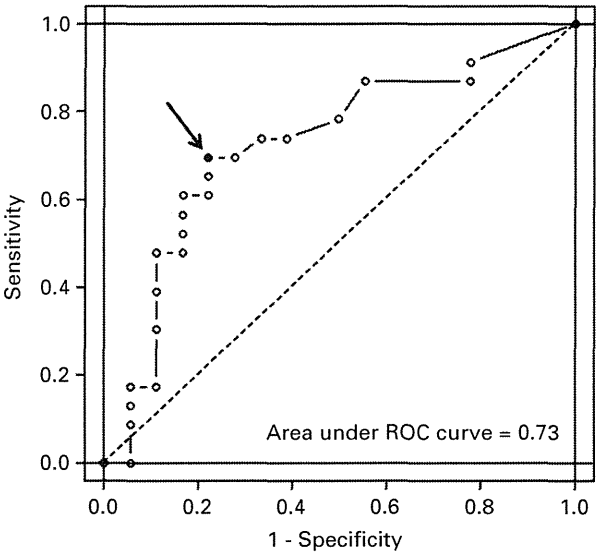

Figure 4. Receiver operating characteristic (ROC) analysis. ROC curve for the ratios of Tregs:CD4$^+$T-cells in identifying patients with aGVHD. The dashed diagonal line represents non-discrimination. Arrow, cutoff ratio at which the sensitivity and specificity resulted in a maximal Youden's index (cutoff ratio, 9%; sensitivity, 69.6%; specificity, 77.8%).

DISCUSSION

In this study, we found that ratios of Treg:CD4$^+$T-cells during the second week after HLA-mismatched HSCT without T-cell depletion accurately predicted the incidence of future aGVHD. Other investigators have used flow cytometry with intracellular staining of FOXP3 to demonstrate a relationship between aGVHD and Treg frequency in peripheral blood. Rezvani *et al.*[11] demonstrated a significant decrease in Treg frequencies at days 30 and 45 when comparing patients with and without aGVHD at the time of Treg sampling, as did Ratajczak *et al.*[14] at a mean of 3 months. Furthermore, Magenau *et al.*[13] assessed Tregs at the onset of aGVHD and demonstrated significant decreases in Treg frequencies in aGVHD patients, with comparison done using samples at GVHD onset and from patients without GVHD, such that the two groups were balanced for the time of acquisition. While their study sampled Tregs more than 4 weeks post transplantation, our study reports significant differences at less than half this time. Although further investigation is needed to determine whether early-stage Treg measurements are possible in other alloHSCT settings, our method under the condition of HLA-mismatched HSCT with rapid hematopoietic reconstitution[15–17]

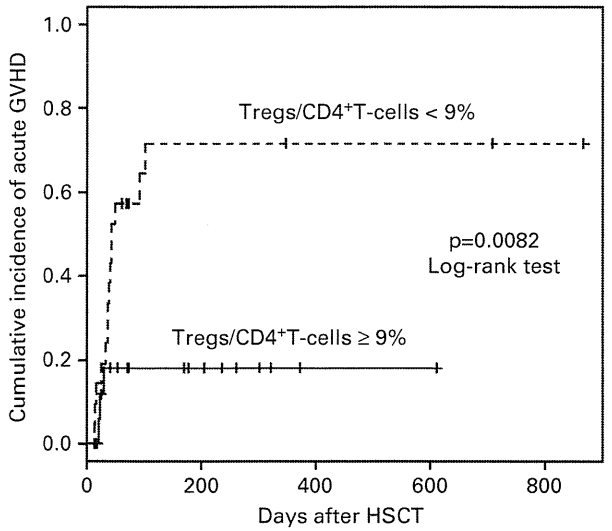

Figure 5. Cumulative incidence of aGVHD. Patients with Treg:CD4$^+$ T-cell ratios $<9\%$ had a significant higher incidence of aGVHD than those with ratios $\geqslant 9\%$ ($P=0.0082$, log-rank test). Measurements were taken in the second week after HSCT.

produced the earliest reported differences in Treg frequency, a finding with practical applications that allows for the prediction of future incidence of aGVHD.

It is a commonly accepted theory that GVHD is initiated in the priming phase, in which donor T-cells activate and proliferate in response to host APCs in secondary lymphoid organs,[21] where Tregs presumably function effectively by suppressing APC function.[22] TNFα is also well known as a central cytokine that peaks immediately after HSCT and stimulates APCs to prime T-cells during this phase.[21,23] Choi *et al.*[24] and Willems *et al.*[25] have both demonstrated that levels of TNFα receptor 1 (a surrogate marker of TNFα) at day 7 correlate with subsequent development of GVHD after myeloablative and nonmyeloablative alloHSCT, respectively. Although a number of studies using animal models have contributed to theories underlying GVHD pathogenesis,[26–30] their finding that this priming phase is limited to a very short duration immediately after HSCT in humans is particularly valuable. The most important point in our study is that conducting investigations at the earliest possible time point after HSCT enabled us to obtain our findings from a very narrow time window. Although it remains unclear whether lower Treg frequencies in peripheral blood reflect lower frequencies in secondary lymphoid organs, integrating our findings and previous studies in which the decline in Treg frequency in peripheral blood was seen during the initial phase of GVHD,[31] it is reasonable to assume that this is indeed the case, and thus that it causes the development of GVHD.

We also found that while absolute numbers of each lymphocyte population did not predict the occurrence of aGVHD, the Treg:CD4$^+$T-cell ratio was the most significant predictor among all other ratios (Table 3). As Tregs can work in cooperation with other cells, including APCs and other T-cells,[22] it is considered rational that ratio rather than absolute number is the relevant factor for predicting aGVHD. However, the reason why the specific ratio of Tregs:CD4$^+$T-cells is the most significant predictor remains uncertain. Although CD4$^+$T-cells recognize MHC class II molecules and have been shown to induce GVHD in a class II-mismatched (class I-matched) murine HSCT model,[32] the importance of CD4$^+$T-cells in GVHD pathogenesis has been demonstrated in fully MHC-mismatched (both class I and class II) murine models[33–35] and even in an HLA class I-mismatched HSCT.[36] Beilhack *et al.*[33] visualized initial proliferation of CD4$^+$T-cells followed by CD8$^+$T-cells in secondary lymphoid organs, and Ewing *et al.*[34] and Yu *et al.*[35] have demonstrated that the activity of CD4$^+$T-cells in the early phase contributes to subsequent development of aGVHD by CD8$^+$T-cells. Accordingly, CD4$^+$T-cells would likely have a leading role during the priming phase of aGVHD, and only then would activation and proliferation of CD8$^+$T-cells proceed. Our observation that both higher CD4$^+$ T-cell:whole T-cell and lower CD8$^+$T-cell:CD4$^+$T-cell ratios in the second week exhibit a significant relationship with aGVHD development does not conflict with these findings, as they both indicate a greater abundance of CD4$^+$T-cells than CD8$^+$T-cells. Furthermore, CD4$^+$T-cells have a particularly direct relationship with Tregs, with CD4$^+$T-cells being the principal targets that Tregs suppress in APC-dependent[37] and -independent[38] manners. Considering this, the high significance of the Treg:CD4$^+$T-cell ratio is reasonable.

Whereas 22 patients developed aGVHD in this study, half of those had grade 1 aGVHD. As previously described,[15] once aGVHD appears in these HLA-mismatched HSCT cases, it inevitably and rapidly progresses to more severe disease, resulting in fatal outcome. All the 11 patients with grade I aGVHD had stage 1 or 2 skin disease at onset. We were therefore obliged to treat them at the earliest time possible, usually within 24 h, with a combination of topical treatment and dose escalation of internal corticosteroid as initial treatment. Additionally, in cases where a skin biopsy was performed, we were unable to delay treatment while waiting for the results, although they would have been helpful for subsequent validation of treatment. Consequently, the disease remained at grade 1 in half of the patients, whereas progression could not be prevented in the other half. It is notable that Treg:CD4$^+$T-cell ratios are able to predict even mild cases of aGVHD, as even grade 1 aGVHD poses a high risk of causing more serious conditions, and should be avoided if possible. In contrast to previous studies,[13,14] we did not observe a significant inverse relationship between aGVHD grade and Treg:CD4$^+$T-cell ratio (data not shown). We attribute this to the early intervention and/or unequal distribution of patients for each grade.

We have demonstrated that patients who developed aGVHD had significantly lower Treg:CD4$^+$T-cell ratios at the second week after HLA-mismatched HSCT, well in advance of clinical aGVHD symptoms. The measurement of Tregs during the second week therefore provides a means to predict the development of aGVHD. Our results suggest that Tregs have a vital role in regulating aGVHD progression, and support the efficacy of early infusions of donor Tregs to prevent GVHD in HLA-haploidentical HSCT.[39] Further studies are needed to confirm whether interventions lead to improved outcomes for patients who show a high risk of aGVHD during the second week post HSCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Expansion of donor-reactive host T cells in primary graft failure after allogeneic hematopoietic SCT following reduced-intensity conditioning

M Koyama[1,2], D Hashimoto[3], K Nagafuji[4], T Eto[5], Y Ohno[6], K Aoyama[1], H Iwasaki[7], T Miyamoto[1], GR Hill[2], K Akashi[1,7] and T Teshima[3]

Graft rejection remains a major obstacle in allogeneic hematopoietic SCT following reduced-intensity conditioning (RIC-SCT), particularly after cord blood transplantation (CBT). In a murine MHC-mismatched model of RIC-SCT, primary graft rejection was associated with activation and expansion of donor-reactive host T cells in peripheral blood and BM early after SCT. Donor-derived dendritic cells are at least partly involved in host T-cell activation. We then evaluated if such an expansion of host T cells could be associated with graft rejection after RIC-CBT. Expansion of residual host lymphocytes was observed in 4/7 patients with graft rejection at 3 weeks after CBT, but in none of the 17 patients who achieved engraftment. These results suggest the crucial role of residual host T cells after RIC-SCT in graft rejection and expansion of host T cells could be a marker of graft rejection. Development of more efficient T cell-suppressive conditioning regimens may be necessary in the context of RIC-SCT.

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Keywords: graft rejection; cord blood transplantation; reduced intensity conditioning

INTRODUCTION

Allogeneic hematopoietic SCT is a curative therapy for various hematological malignant tumors, BM failure and congenital immune and metabolic disorders. The success of SCT is highly dependent on the suppression of the recipient's immune system to prevent graft rejection by host immunocompetent cells. Progress on SCT has minimized the rate of graft rejection by the selection of HLA-matched donors, the use of myeloablative conditioning regimens, and infusion of large numbers of hematopoietic stem cells (HSCs).[1–6] However, the incidence of graft rejection is again increasing as the wider application of allogeneic SCT with the increasing use of HLA-mismatched donors, reduced-intensity conditioning (RIC), which could retain host immune cells and HSCs compared with myeloablative conditioning, and cord blood that contains small numbers of HSCs compared with G-CSF-mobilized peripheral blood or BM.[7–9]

Graft failure or graft rejection has been defined as either a lack of initial engraftment of donor cells or loss of donor cells after initial engraftment.[10] Rejection is a major cause of graft failure and is caused by recipient T cells, natural killer (NK) cells, or Abs.[10–15] An increase in number of residual host T cells is associated with graft rejection in pediatric allogeneic transplantation.[16] HLA-C mismatch with NK epitope mismatching in the rejection direction are associated with higher rates of graft rejection after HLA-mismatched transplantation.[17,18] Positive serum crossmatch is predictive for graft failure in HLA-mismatched allogeneic SCT.[19,20]

Thus, the fate of transplanted donor HSCs, namely engraftment or rejection is determined by the competition between donor-derived and residual host-derived HSCs (stem cell competition) and by the competition between donor-derived and host-derived immune competent cells, such as T cells, NK cells and B cells (immunological competition).[11–13]

We herein investigated the process of host T cell-mediated immunological graft rejection in a mouse model of RIC-SCT using sublethal irradiation conditioning[21] and in patients who underwent cord blood transplantation (CBT) following RIC (RIC-CBT) that represents a higher risk for graft rejection than other types of allogeneic SCT.

MATERIALS AND METHODS

A mouse model of BMT

Female C57BL/6 (B6: H-2^b, CD45.2$^+$), B6D2F1 (H-2$^{b/d}$, CD45.2$^+$), DBA/2 (H-2^d, CD45.2$^+$), B6-Ly5a (H-2^b, CD45.1$^+$) and B6.FVB-Tg (Itgax-DTR/EGFP) 57Lan/J (B6.CD11c-DTR, H-2^b) mice were purchased from Charles River Japan (Yokohama, Japan), the Jackson Laboratories (Bar Harbor, ME, USA), and the Animal Resources Center (Perth, Western Australia, Australia). B6.CD11c-DTR × DBA/2F1 mice were generated by breeding B6.CD11c-DTR mice with DBA/2 mice. All experiments involving animals were performed under the auspices of the Institutional Animal Ethics Committee.

Following 6 or 10 Gy TBI (X-ray), B6 mice were injected with 5×10^6 NK cell-depleted BM cells from allogeneic B6D2F1 or syngeneic B6-Ly5a donor mice on day 0. NK-cell depletion of BM cells was performed using CD49b (DX5) microbeads and the autoMACS (Miltenyi Biotec Japan, Tokyo, Japan) according to the manufacturer's instructions. Mice were housed in sterilized microisolator cages and received autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT. For donor DC depletion, all recipients were injected with 100 ng diphtheria toxin (DT) i.p. every other day from day 0 until the end of experiments.

[1]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Science, Fukuoka, Japan; [2]Bone Marrow Transplantation Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland, Australia; [3]Department of Hematology, Hokkaido University Graduate School of Medical Science, Sapporo, Japan; [4]Department of Hematology, Kurume University, Fukuoka, Japan; [5]Department of Hematology, Hamanomachi General Hospital, Fukuoka, Japan; [6]Internal medicine, Kitakyushu Municipal Medical Center, Kitakyushu, Japan and [7]Center for Cellular and Molecular Medicine, Kyushu University Graduate School of Medical Science, Fukuoka, Japan.
Correspondence: Dr T Teshima, Department of Hematology, Hokkaido University Graduate School of Medicine, N15 W7, Kita-Ku, Sapporo 060 8638, Japan.
E-mail: teshima@med.hokudai.ac.jp
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After transplant, complete blood counts were performed using a Celltac MEK-6358 (Nihon Kohden, Tokyo, Japan). Donor cell chimerism was evaluated weekly in the peripheral blood. mAbs used were CD4, CD8, CD45.1, CD45.2, TCRβ, H-2 K^b and H-2 K^d (BD Biosciences, San Diego, CA, USA). Donor and host cells in a BDF1 → B6 BMT were identified as H-2 K^{b+} H-2 K^{d+} and H-2 K^{b+} H-2 K^{d-}, respectively. In a B6-Ly5a → B6 syngeneic BMT, CD45.1 and CD45.2 were used as donor and host cell-specific markers, respectively. Complete donor cell engraftment, mixed donor and host chimerism, and rejection were defined as >95%, 5–95% and <5% donor chimerism of WBCs in peripheral blood, respectively. For intracellular IFN-γ staining, cells were incubated for 4 h with Leukocyte Activation Cocktail (BD Biosciences) at 37 °C. Then, the cells underwent permeabilization with BD Cytofix/cytoperm solution (BD Bioscience) and were stained with FITC-conjugated IFN-γ mAbs (BD Bioscience). Dead cells were identified as 7-amino-actinomycin D (7-AAD; BD Biosciences)-positive cells. The cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using a Flow Jo software (Tree Star, San Carlos, CA, USA).

CTL assays

Fourteen days after BMT, T cells were separated from BM of the bilateral tibias and femurs or spleen using Thy1.2 (CD90.2) microbeads and the autoMACS according to the manufacturer's instructions. The percentage of $CD8^+$ cells in this fraction was determined by flow cytometry and counts were normalized for $CD8^+$ cell numbers. CTL assays were performed as previously described.[22] Briefly, tumor targets, 2×10^6 DBA/2-derived mastocytoma cell line P815 (H-2^d) or B6-derived lymphoma cell line EL4 (H-2^b), were labeled with 100 μCi of ^{51}Cr sodium salt (Amersham Biosciences, Tokyo, Japan) for 2 h. After washing three times, the labeled targets were plated at 10^4 cells per well in U-bottom plates (Corning-Costar Corp., Cambridge, MA, USA). Allogeneic T-cell preparations, as described above, were added to quadruplicate wells at varying effector-to-target ratios and incubated for 4 h. Maximal and background release were determined by adding 1% SDS or media alone to the targets, respectively. ^{51}Cr activity in the supernatants collected 4 h later were determined using a Wallac 1470 WIZARD Gamma Counter (Wallac Oy, Turku, Finland), and lysis was expressed as a percentage of maximum: percentage of specific lysis = 100 (sample count − background count/maximum count − background count).

For CTL assay against hematopoietic cells, $CD8^+$ cells were separated from BM of the bilateral tibias and femurs using CD8 microbeads and the midiMACS 21 days after BMT. Donor and host-derived hematopoietic target cells were isolated from BM of naïve B6-Ly5a and B6D2F1 mice by depleting lineage committed cells using biotin-conjugated CD3, CD5, Ter119, Gr1, Mac1, B220 Abs and BD IMag Streptavidin Particles Plus-DM (BD Bioscience). The lineage negative targets were plated at 3×10^3 cells per well in 96 U-bottom plates with 1.5×10^5 $CD8^+$ cells and incubated for 4 h. After incubation, the cells were collected and stained with target marker (H2Dd or CD45.1), lineage, Sca-1, c-kit, Annexin V and SYTOX blue stain (Invitrogen, Palsley, UK) for FACS analysis (Fortessa LSD, BD Bioscience).[23]

Patients

We retrospectively analyzed data from 24 adult patients who underwent RIC-CBT at Kyushu University Hospital, Hamanomachi Hospital and Kitakyushu Medical Center between August 2003 and February 2007 and survived more than 28 days post transplant. Patients who received the second CBT for rejection after primary transplant were excluded. This study was approved by the institutional review board of each participating institute.

Transplantation procedure and definitions

Cord blood units were obtained from the Japan Cord Blood Bank Network. Serological typing for HLA-A, -B and -DR Ags of cord blood units and patients' blood samples was performed. HLA-mismatch in the graft-versus-host vector was defined when the recipient's Ags were not shared by the donor, while mismatch in the host-versus-graft vector was defined as when the donor's Ags were not shared by the recipient. RIC regimens were defined as previously reported.[24–26] Regimens used were fludarabine 150–180 mg/m^2 with either CY 60 mg/kg, BU 8 mg/kg or melphalan 80–140 mg/m^2 with 4 Gy TBI. The prophylaxis regimens for GVHD were CYA or tacrolimus alone, CYA plus MTX or CYA plus mycophenolate mofetil. Risk status at transplantation was categorized as either standard risk or high risk. Standard-risk diseases included acute leukemia in first CR, CML in first chronic phase and refractory anemia of myelodysplastic syndrome. Other diseases were categorized as high-risk disease. Graft failure was defined as failure of the ANC to surpass 0.5×10^9/L for 3 consecutive days before relapse, second transplantation or death.[27] Chimerism of WBCs was assessed 3 weeks after CBT by using FISH in sex-mismatched donor-recipient pairs or PCR for variable numbers of tandem repeats in sex-matched pairs at a sensitivity of 10%.[28]

Statistical analysis

Mann–Whitney *U*-tests were used to analyze cell counts and clinical scores. We defined $P<0.05$ as statistically significant. The values are described with s.e.

RESULTS

Intensity of TBI impacts donor cell engraftment

We first examined the impact of conditioning intensity on donor cell engraftment in a murine MHC-mismatched B6D2F1 (H-$2^{b/d}$) → B6 (H-2^b) model (F1 → P) of BMT.[21] In this model, donor T cells are tolerant of the recipient MHC, but donor NK cells not expressing H-2^b-specific Ly49C/I inhibitory receptors and bearing instead H-2^d-specific Ly49A/G2 receptors can be activated to kill the recipient's targets.[29] In contrast, recipient T cells are activated by the donor-derived alloantigens, whereas recipient NK cells are not reactive to the donor's targets. Therefore, graft rejection is primarily mediated by donor-reactive host T cells in this model. Donor NK cells that can compete with host T cells were depleted from the donor inoculum. B6 mice were exposed to 6 or 10 Gy TBI and then intravenously injected with 5×10^6 NK cell-depleted BM cells from B6D2F1 mice or B6-Ly5a mice on day 0. It has been shown that conditioning with ⩽7 Gy TBI is not lethal in this strain combination.[21] After BMT, donor cell chimerism was evaluated in the peripheral blood weekly. WBC counts recovered rapidly to the normal level by 4 weeks after syngeneic BMT and allogeneic BMT following 10 Gy TBI, but not after allogeneic BMT following 6 Gy TBI (Figure 1a). Complete donor chimerism was rapidly achieved after allogeneic BMT following 10 Gy TBI, whereas donor chimerism never exceeded 10% after allogeneic BMT following 6 Gy TBI (Figure 1b). In contrast, graft failure did not develop in the syngeneic recipients with 6 Gy TBI, confirming that the graft rejection in the allogeneic recipients with 6 Gy TBI resulted from alloreactivity not insufficient numbers of stem cells.

Temporal expansion of host T cells in the peripheral blood in mice experiencing graft rejection

We enumerated donor and host T cells separately after BMT. The number of donor T cells (H-2 K^{b+} H-2 K^{d+} $TCR\beta^+$) in peripheral blood increased rapidly in the allogeneic recipients with 10 Gy TBI and the syngeneic recipients with 6 Gy TBI but not in the allogeneic recipients with 6 Gy TBI (Figure 1c). Such an increase of donor T cells in these animals is likely mediated by homeostatic proliferation in lymphopenia after BMT. Residual host T cells (H-2 K^{b+} H-2 K^{d-} $TCR\beta^+$) accounted for only 10% of total T cells at 6 weeks after BMT in the allogeneic recipients with 10 Gy TBI (Figure 1d). In contrast, host T cells markedly increased with a steep peak at 3 weeks after BMT in the allogeneic recipients with 6 Gy TBI. Such a surge of residual host T cells which consisted of 99% of total T cells was not seen in either of the allogeneic recipients with 10 Gy TBI or the syngeneic recipients with 6 Gy TBI. Given that those graft rejections were not caused by insufficient number of stem cells (Figure 1b), we hypothesized that the expansion of host T cells in the allogeneic recipients with 6 Gy TBI could be responsible for primary graft rejection.

Host T-cell expansion is associated with early rejection of donor BM graft

We examined whether the activated host T cells could expand not only in peripheral blood but also in hematopoietic organs where

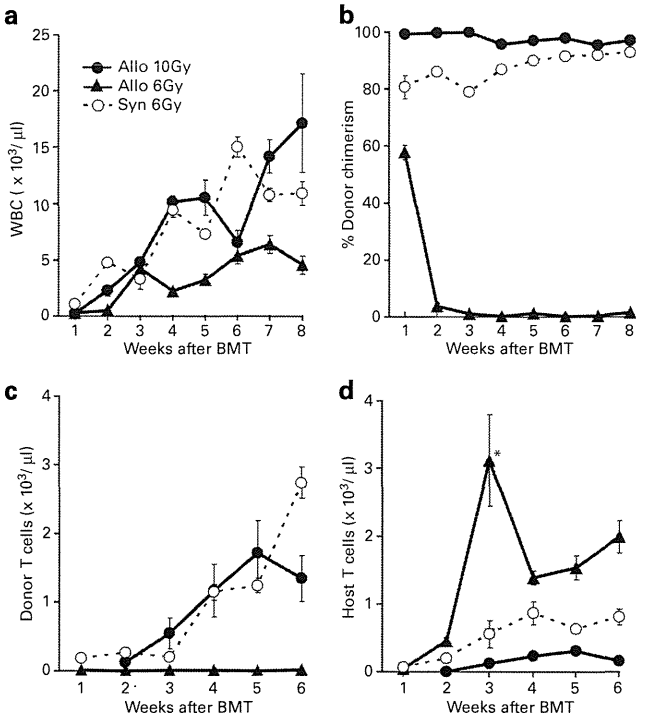

Figure 1. The impact of TBI doses on donor cell engraftment. B6 mice were exposed to 10 Gy TBI ($n=6$, closed circle with solid line) or 6 Gy TBI ($n=9$; closed triangle with solid line), and i.v. injected with 5×10^6 NK cell-depleted BM cells from B6D2F1 mice on day 0. In syngeneic controls, B6 mice were transplanted with cells from B6-Ly5a mice following 6 Gy TBI ($n=6$, open circle with broken line). Numbers **(a)** and donor chimerism **(b)** of WBCs after BMT. Numbers of donor (**c**) and host (**d**) T cells. Donor T cells were H2-K^{b+} H-2 K^{d+} $TCR\beta^+$ cells and host T cells were H2-K^{b+} H-2 K^{d-} $TCR\beta^+$ cells. Results from three similar experiments were combined ($n=6$–9 per group). *$P<0.01$ compared with 2 and 4 w. Data are shown as the mean ± s.e.

donor graft cells migrate. B6 recipients irradiated with 6 Gy TBI were i.v. injected with 5×10^6 NK cell-depleted BM cells from allogeneic B6D2F1 mice or syngeneic B6-Ly5a mice. Two weeks later when donor chimerism has already been lost in allogeneic recipients (Figure 1b), cells were isolated from BM and spleen to analyze chimerism and proliferation of host T cells. In allogeneic animals, host-derived cells (H2-K^{b+} H-2 K^{d-}) exceeded 99% in BM (99.6% ± 0.34%) and spleen (99.6% ± 0.30%), whereas it was 44.7% ± 22.1% in the BM and 48.4% ± 32.2% in the spleen of syngeneic recipients (data not shown). Host-derived IFN-γ^+ $CD8^+$ T cells markedly expanded in the BM and spleen from allogeneic recipients compared with those in syngeneic recipients both at 2 weeks (Figure 2a) and 3 weeks after BMT (Figure 2b). In contrast, host-derived IFN-γ^+ $CD4^+$ T cells were modestly increased in the spleen 2 weeks after BMT and in the BM 3 weeks after BMT in allogeneic recipients. Frequencies of host-derived IFN-γ^+ T cells were also increased in BM (Figure 2c). We next evaluated the cytolytic activity of those host $CD8^+$ T cells with respect to donor targets. BM T cells were isolated from allogeneic recipients treated with 6 Gy TBI 2 weeks after BMT. We confirmed with a flow cytometric analysis that more than 99% of these T cells were host-derived (data not shown). These T cells exhibited CTL activity against donor-type P815 (H-2^d) targets but not against donor-type EL4 (H-2^b) targets (Figure 2d). Similar results were obtained when splenic T cells were used (data not shown). Furthermore, the $CD8^+$ T cells isolated from BM of graft-rejected recipients targeted and induced cell death of donor-type HSCs (lineage$^-$ c-Kit^+ $Sca\text{-}1^+$ cells) but not host-type HSCs as Annexin V^+ $SYTOX^+$ cells (Figure 2e). These results suggest that the expansion of donor-reactive host T cells in hematopoietic tissue including BM precedes the transient increase of host T cell in peripheral blood in the allogeneic recipients who experienced primary graft rejection.

Donor DC depletion abolished the transient host T-cell expansion and improved donor chimerism partially

Next we examined if donor cell rejection could be prevented when host T-cell activation and expansion were inhibited. Donor DCs were expected as the main population to activate host T cells.[30] B6 recipients irradiated with 6 Gy TBI were transplanted with 5×10^6 NK cell-depleted BM cells from allogeneic wild-type B6D2F1 or B6.CD11c-DTR × DBA/2F1 mice. All recipients were injected with 100 ng DT i.p. every other day from day 0. We evaluated kinetics of host T-cell expansion and donor chimerism in peripheral blood every week. Host T-cell expansion was suppressed (Figure 3a) and the donor chimerism at 3 weeks after BMT was increased in donor DC-depleted recipients compared with DC-repleted controls (Figure 3b).

Host T cell surge in the patients who resulted in primary graft rejection following RI-CBT

We next investigated clinical relevance of such a "surge" of host T cells in rejection in adult patients who underwent RIC-CBT retrospectively. We focused on RIC-CBT, because primary graft rejection was rare after BMT or PBSC transplantation.[9] In 24 adult patients, who underwent RIC-CBT, 17 patients achieved engraftment at a median of 22 days (range, 13–31 days), while primary graft rejection occurred in 7 patients (Table 1). The number of infused cells, $CD34^+$ cells and HLA disparity were evenly distributed between the groups (Table 1). We clearly observed a surge of lymphocytes in 4 out of 7 patients who experienced primary graft rejection (Figure 4a), but not in patients with engraftment (Figure 4b). The chimerism analysis at 3 weeks after CBT demonstrated that expanded lymphocytes were host-derived (Figure 4c). One out of 4 patients with the transient lymphocyte increase had aplastic anemia as the original disease and the other three patients received single course of cytotoxic chemotherapy for the original malignant diseases before CBT. A flow cytometric analysis of these host lymphocytes was performed in one representative patient with rejection; 85% of the cells were $CD8^+$ T cells (data not shown).

DISCUSSION

Graft rejection is mediated by infusion of insufficient numbers of HSCs and/or humoral and cellular immunological mechanisms, involving anti-HLA Abs and donor-reactive host T cells and NK cells.[10,31] Although standard myeloablative conditioning is usually sufficient to suppress donor-reactive host T cells and permits donor cell engraftment, RIC leaves larger numbers of residual host immunocompetent cells than myeloablative conditioning. However, the impacts of RIC on the kinetics of host T cells in the context of graft rejection have not been well delineated.

We first evaluated effects of nonmyeloablative conditioning on host T cells in a mouse F1 → P model with donor NK-cell depletion, where recipient T cells can recognize donor alloantigens, while donor T cells cannot recognize recipient alloantigens. This model thus represents a simplified model that allows us to examine the effects of conditioning intensity on the ability of anti-donor host T cells to mediate graft rejection independent of the presence of donor-derived T cells and NK cells, although mechanisms of clinical rejection are more complex, involving donor T and NK-cell responses. In this model, the conditioning with 6 Gy TBI resulted in primary graft rejection, whereas syngeneic BMT with the same number of BM cells following 6 Gy TBI achieved donor-dominant chimerism, indicating the T cell-mediated graft rejection, not due

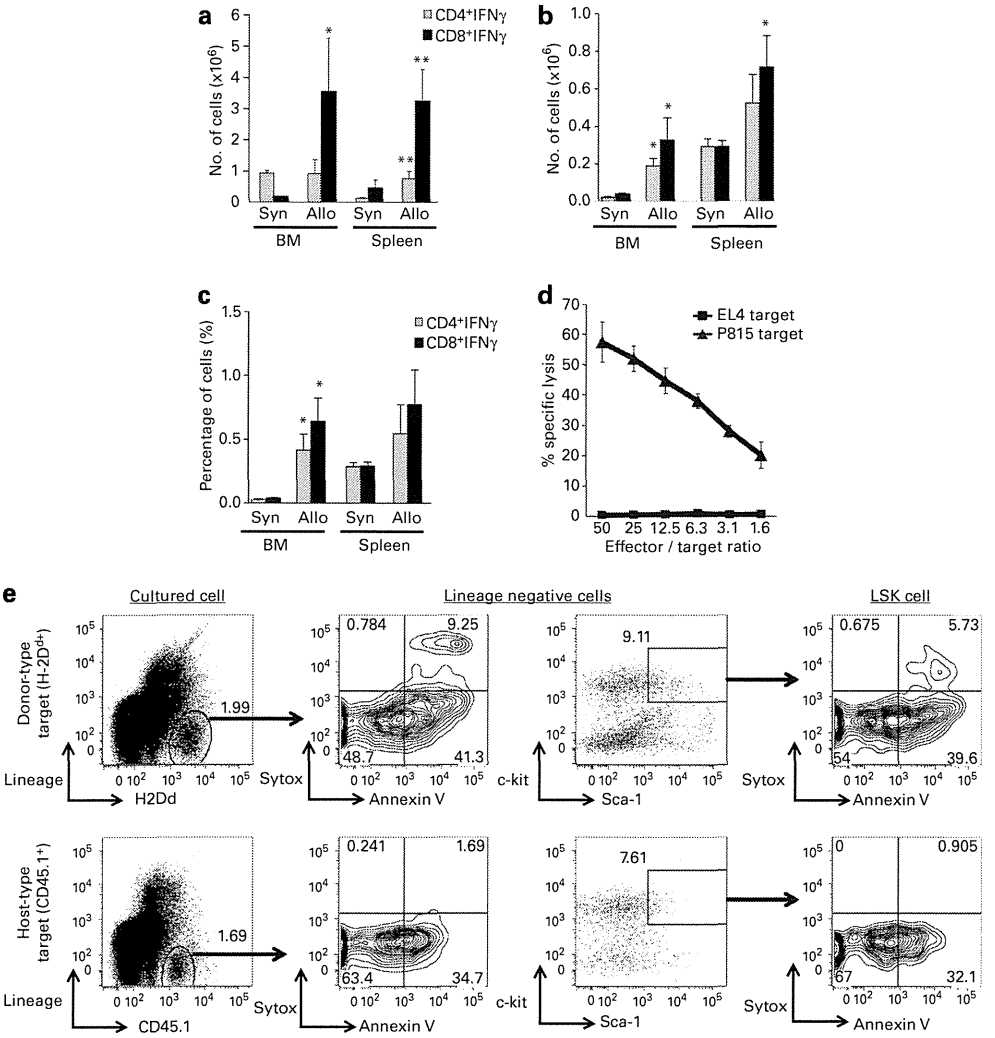

Figure 2. Expansion of anti-donor cytotoxic host T cells. B6 mice received 6 Gy TBI and then injected i.v. with 5×10^6 NK cell-depleted BM cells from B6D2F1 (allo) or B6-Ly5a mice (syn). Splenocytes and BM cells were isolated from the bilateral tibias and femurs 2 and 3 weeks after BMT. (**a**, **b**) The absolute numbers of IFNγ^+ CD4$^+$ cells (gray bar) and IFNγ^+ CD8$^+$ cells (black bar) in the BM and spleen 2 weeks (**a**) and 3 weeks after BMT (**b**). (**c**) Frequencies of IFNγ^+ CD4$^+$ cells (gray bar) and IFNγ^+ CD8$^+$ cells (black bar) in the BM and spleen 3 weeks after BMT. (**d**, **e**) BM T cells were cultured with P815 (H-2^d) targets or EL4 (H-2^b) targets at varying effector-to-target ratios (**d**), or with lineage negative BM cells from naïve donor-type B6D2F1 (H-2$^{b/d}$) mouse or host-type B6-Ly5a mice (CD45.1$^+$) (**e**) to determine cell cytotoxicity. Data shown are representative of two replicate experiments and mean ± s.e. ($n = 4$–10 per group). *$P < 0.01$, **$P < 0.0001$ compared with syn.

to an insufficient number of stem cells in the graft. The conditioning with 10 Gy TBI resulted in donor-dominant chimerism (>98% donor cells in WBC) 6 weeks after BMT, although there were some residual host-derived T cells. However, these residual T cells did not induce graft rejection probably due to their function being impaired by 10 Gy TBI.

We found graft rejection was associated with an increase in numbers of host T cells in the peripheral blood three weeks after BMT and expansion of cytotoxic CD8$^+$ T cells in the hematopoietic tissues, BM and spleen. These results correspond with the previous experimental studies that CD8-deficient recipient mice were superior for engraftment[32,33] and that preconditioning with anti-CD8 mAb enhanced allogeneic engraftment.[34]

Given that host T cells selectively targeted donor-type cells (Figure 2), these host T cells should recognize donor-type MHC and minor histocompatibility antigens directly presented on donor APCs or indirectly presented on host APCs. Although the dominant pathway of allorecognition by host T cells is difficult to be discriminated in our model, these findings lead us to the hypothesis that the inhibition of donor alloantigen presentation to host T cells could permit donor cell engraftment.[30,35] Donor DC depletion by DT administration suppressed the transient host T-cell expansion and delayed graft rejection with transient mixed chimerism, but failed to maintain sustained engraftment. This is probably due to the presence of other subsets of donor-derived APCs than CD11c$^+$ DCs, as well as insufficient DC depletion after multiple injections of DT as has been reported.[36] Nonetheless, our results are consistent with a previous study showing that donor APCs, not host APCs, particularly DCs have a critical role in eliciting BM rejection.[30] However, evidence suggesting the causative role of donor DCs in BM rejection is still limited; therefore, there is also the possibility that the other components of donor or host cells have a role in inducing rejection.[35,37] Nonetheless, our results suggest that primary graft rejection is at least partly mediated by host T cells stimulated by donor DCs.

We then evaluated the relevance of the overshoot of host T cells observed after MHC-mismatched RIC-SCT in a mouse model in patients experiencing graft rejection after HLA-mismatched RIC-CBT, as rejection is more frequent after CBT.[38] Four out of the seven patients who experienced graft rejection had the temporal

Table 1. Characteristics of patients, donors and transplantation regimens

Variable	*Graft rejection*	*Engraftment*
No. of patients	7	17
Age (years), median (range)	63 (29–65)	40 (23–65)
Risk status (standard risk/ high risk)	4/3	4/13
Primary diseases		
Hematological malignancies	6	16
Aplastic anemia	1	1
No. of infused nuclear cells, median (range), 1×10^7 per kg	3.06 (2.05–3.20)	2.94 (1.69–-6.02)
No. of infused $CD34^+$ cells, median (range), 1×10^5 per kg	0.87 (0.42–1.43)	1.00 (0.70–1.83)
HLA-mismatch in the graft-versus-host vector		
0	0	0
1	4	4
2	3	9
More than 3	0	4
GVHD prophylaxis		
CYA or FK506 alone	0	5
CYA + sMTX	6	6
CYA + MMF	1	6
Day of engraftment, median (range)	NA	22 (13–31)

Abbreviations: Flu, fludarabine; LPAM, Melphalan; CL, cladribine; sMTX, short-term MTX; MMF, mycophenolate mofetil; NA, not applicable.

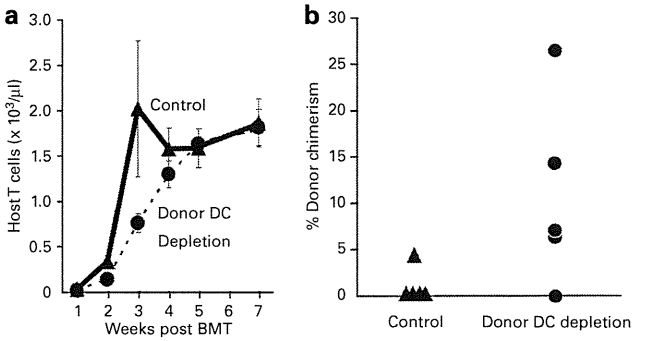

Figure 3. Donor DC depletion improves donor chimerism while abolishing the transient host T-cell increase. B6 recipients irradiated with 6 Gy were transplanted with 5×10^6 NK cell-depleted BM cells from allogeneic wild-type B6D2F1 or B6.CD11c-DTR × DBA/2F1 mice. All recipients were injected with 100 ng DT i.p. every other day from day 0. Time-course of the numbers of host T cells (**a**) and chimerism at 3 weeks after BMT (**b**) in peripheral blood are shown. Data are shown as the mean ± s.e. ($n=5$ per group).

increase in numbers of host-derived lymphocytes 3 weeks after RIC-CBT, similar to the observation in the murine model. We confirmed that more than 80% of these lymphocytes were $CD8^+$ T cells in one patient. Our results are consistent with previous clinical studies demonstrating the presence of host-derived, anti-donor T cells in recipients who experienced graft rejection after CBT.[39–43] In addition to lower numbers of HSCs infused in CBT, lower numbers and impaired function of CB T cells and/or APCs may be associated with host T-cell expansion and graft rejection after CBT.[44–48] Further studies are also required to evaluate naïve versus memory phenotypes of host T cells expanded in graft

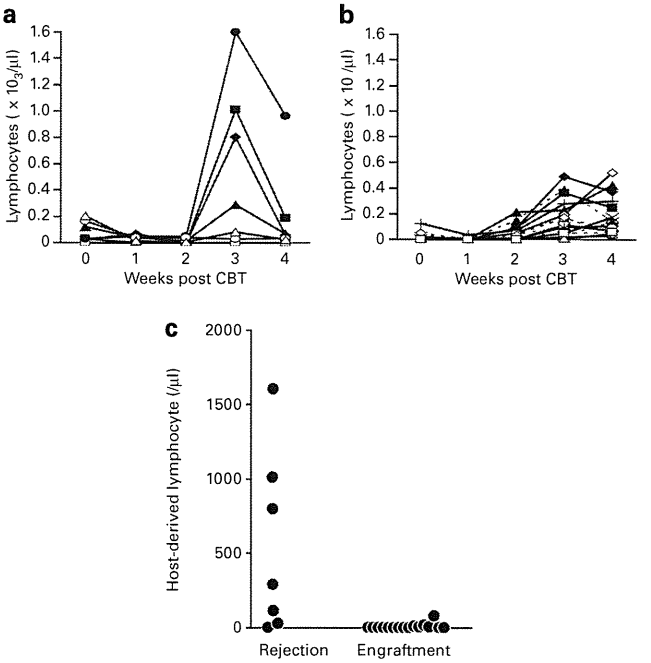

Figure 4. A surge of host lymphocytes in patients with graft rejection after RI-CBT. Numbers of lymphocytes in peripheral blood of patients with rejection (**a**) and those without rejection (**b**) after RI-CBT. (**c**) Numbers of host-derived lymphocytes 3 weeks post transplant were the products of numbers of lymphocytes and percentage of host-derived cells in peripheral blood.

rejection to determine a role of host memory T cells, which had been sensitized by prior transfusion.

In conclusion, our results suggest a crucial role of host-derived anti-donor T cells in primary graft rejection after RIC-SCT and lead us to speculate that developing conditionings to suppress host T cells efficiently is urgently required for RIC-CBT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: MK, DH, KA and YS performed the research; MK, KN, TE, YO and TM analyzed patient data; MK and TT designed the research study and wrote the paper.

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Mammalian Target of Rapamycin Inhibitors Permit Regulatory T Cell Reconstitution and Inhibit Experimental Chronic Graft-versus-Host Disease

Haruko Sugiyama [1], Yoshinobu Maeda [1,*], Hisakazu Nishimori [1], Yoshiko Yamasuji [1], Ken-ichi Matsuoka [1], Nobuharu Fujii [1], Eisei Kondo [1], Katsuji Shinagawa [1], Takehiro Tanaka [2], Kengo Takeuchi [3], Takanori Teshima [4], Mitsune Tanimoto [1]

[1] *Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan*
[2] *Department of Pathology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan*
[3] *Pathology Project for Molecular Target, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan*
[4] *Department of Hematology, Hokkaido University, Hokkaido, Japan*

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A B S T R A C T

Chronic graft-versus-host disease (GVHD) remains a major late complication of allogeneic bone marrow transplantation (BMT). In a previous study, impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD using MHC class II-deficient (H2-Ab1 KO) B6 into C3H model and $CD4^+$ T cells isolated from chronic GVHD mice caused chronic GVHD when administered into the secondary recipients. In this study, we evaluated the kinetics of regulatory T cell (Treg) reconstitution in wild type B6 into C3H model. After myeloablative conditioning, host Tregs disappeared rapidly, followed by expansion of Tregs derived from the donor splenic T cell inoculum. However, the donor splenic T cell–derived Treg pool contracted gradually and was almost completely replaced by newly generated donor bone marrow (BM)-derived Tregs in the late post-transplantation period. Next, we compared the effects of cyclosporine (CSA) and mammalian target of rapamycin (mTOR) inhibitors on Treg reconstitution. Administration of CSA significantly impaired Treg reconstitution in the spleen and thymus. In contrast, BM-derived Treg reconstitution was not impaired in mTOR inhibitor-treated mice. Histopathological examination indicated that mice treated with CSA, but not mTOR inhibitors, showed pathogenic features of chronic GVHD on day 120. Mice treated with CSA until day 60, but not mTOR inhibitors, developed severe chronic GVHD followed by adoptive transfer of the pathogenic $CD4^+$ T cells isolated from H2-Ab1 KO into C3H model. These findings indicated that long-term use of CSA impairs reconstitution of BM-derived Tregs and increases the liability to chronic GVHD. The choice of immunosuppression, such as calcineurin inhibitor-free GVHD prophylaxis with mTOR inhibitor, may have important implications for the control of chronic GVHD after BMT.

INTRODUCTION

Chronic graft-versus-host disease (GVHD) is the most serious late complication after allogeneic hematopoietic stem cell transplantation, but the pathophysiology and treatment strategy of chronic GVHD remain poorly defined [1-3]. GVHD prophylaxis using calcineurin inhibitors, such as cyclosporine (CSA) and tacrolimus, reduces the expansion of effector T cells by blocking interleukin (IL)-2 and prevents acute GVHD, but fails to reduce chronic GVHD [4,5]. Administration of CSA for up to 24 months, longer than the standard 6 months of CSA, also did not decrease the risk of chronic GVHD [6]. Several studies have indicated that the efficacy and safety of mammalian target of rapamycin (mTOR) inhibitor, rapamycin (RAPA), in refractory chronic GVHD patients [7-10]. However, a recent randomized trial showed that the combination of RAPA and tacrolimus as GVHD prophylaxis failed to reduce chronic GVHD compared with tacrolimus and methotrexate [11].

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) have been shown to play an important role in the establishment of tolerance between recipient tissues and donor-derived immunity. A series of animal studies indicated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells [12-14]. Based on the role of Tregs in the prevention of GVHD and on their dependence on IL-2, there is considerable concern regarding the impact of blocking IL-2 signaling or IL-2 production by the immunosuppressive agents used for prophylaxis of GVHD. Zeiser et al. reported that Tregs showed relative resistance to RAPA as a result of reduced usage of the mTOR pathway and functional phosphatase and tensin homolog, a negative regulator of the phosphatidyl-inositol 3-kinase/Akt/mTOR pathway in Tregs

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* Correspondence and reprint requests: Yoshinobu Maeda, MD, PhD, Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Shikata-cho 2-5-1, Kita-ku, Okayama city, Okayama 700-8558, Japan.
E-mail address: yosmaeda@md.okayama-u.ac.jp (Y. Maeda).

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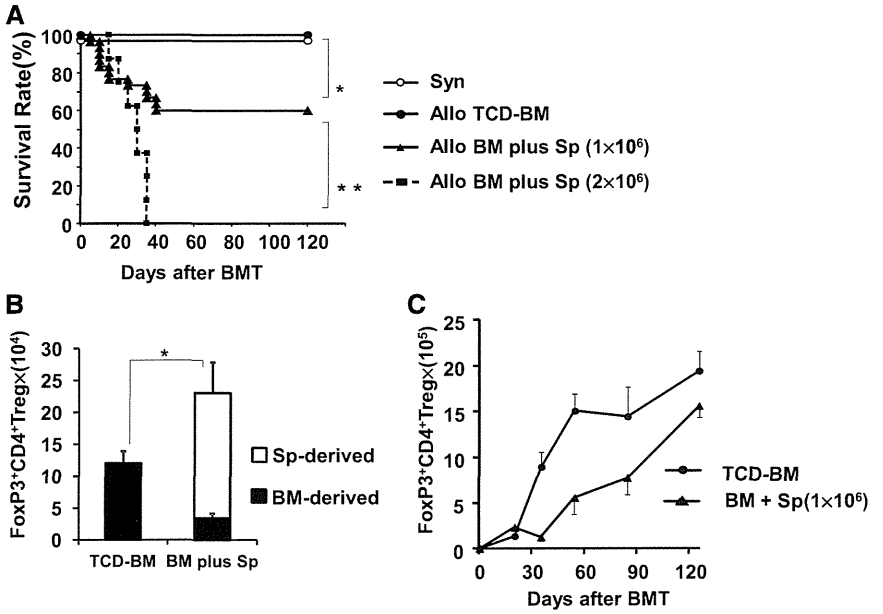

Figure 1. Regulatory T cell reconstitution after allogeneic BMT. Lethally irradiated C3H (H-2^k) recipient mice received 10 × 10^6 T cell–depleted bone marrow (TCD-BM) cells from B6.Ly-5a (H-2^b,CD45.1) mice with/without 1 to 2 × 10^6 spleen cells from B6 (H-2^b,CD45.2) mice. The syngeneic group received transplantation from C3H mice. (A) Survival: the recipients of allogeneic BM plus 1 × 10^6 spleen cells (BM plus Sp cells) showed a survival rate of 60% by day 120. Open circle, syngeneic; closed circle, TCD-BM cells only; triangle, -with 1 × 10^6 spleen cells; square, -with 2 × 10^6 spleen cells. (B) Origin of $CD4^+Foxp3^+$ Treg in the spleen on day 21 post transplantation: $CD45.2^+$ splenic T cell–derived (white bars) and $CD45.2^-$ BM–derived (black bars) are shown. (C) The absolute numbers of Treg in the recipients of BM plus Sp cells (triangles) and TCD-BM (closed circles) are shown. Each group consisted of 7 to 25 mice. The means (±SE) of each group are shown. Data are from a representative of at least 3 independent experiments. $^*P < .05$; $^{**}P < .01$.

compared with conventional T cells [15]. In contrast to CSA, RAPA allowed expansion of adoptively transferred Treg cells and led to reduction of alloreactive T cell expansion when animals received Treg treatment in combination with RAPA. They also showed that a combination of RAPA plus IL-2 increased both expansion of donor natural Tregs and conversion of induced Tregs from donor conventional T cells, and suppressed acute GVHD [16]. These animal data suggest that RAPA and CSA have differential effects on peripheral Tregs after bone marrow transplantation (BMT).

IL-2 signaling is pivotal for Treg homeostasis in the periphery and is also essential for naturally occurring Treg development in the thymus [17-19]. T cell repopulation after BMT is composed of 2 subsets: T cells derived from the donor splenic T cell inoculum and newly arising T cells from bone marrow (BM) inoculum. It has been shown that Tregs from the former pathway play an important role in acute GVHD, whereas, no previous study evaluated whether use of CSA for an extended period affects donor BM-derived Treg generation. We hypothesized that BM-derived Tregs comprise the long-term peripheral Treg pool and that CSA, but not mTOR inhibitors, causes impaired BM-derived Treg reconstitution, which has a negative effect on chronic GVHD. In the present study, we therefore evaluated effects of different immunosuppressants on 2 distinct Treg expansion reconstitution pathways and on the development of chronic GVHD.

MATERIALS AND METHODS

Mice

Female C57BL/6 (B6: H-2^b, $CD45.2^+$) and C3H/HeN (C3H: H-2^k) mice were purchased from Charles River Japan (Yokohama, Japan) or from the Okayama University mouse colony (Okayama, Japan). B6-Ly5a (H-2^b, $CD45.1^+$) and C3.SW (H-2^b, $CD45.2^+$) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II-deficient H2-$Ab1^{-/-}$ mice (B6.129-*H2-Ab1*^{tm1Gru} N12) were from Taconic Farms (Germantown, NY) [20]. Mice between 8 and 18 weeks of age were maintained under specific pathogen-free conditions and received normal chow and hyperchlorinated drinking water after transplantation. All experiments involving animals were approved by the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

BMT

Mice underwent transplantation according to the standard protocol described previously [21,22]. Briefly, recipient mice received 2 split doses of either 500 cGy (allogeneic C3H and C3.SW recipients) or 650 cGy (syngeneic B6 recipients) total-body irradiation (TBI) 3 to 4 hours apart. Recipients were injected with 10 × 10^6 T cell–depleted bone marrow (TCD-BM) cells plus 1 or 2 × 10^6 whole spleen cells from B6 donors. [*H2-Ab1*$^{-/-}$ → C3H] chimeras were produced by reconstituting lethally irradiated C3H mice with 5 × 10^6 TCD-BM cells from *H2-Ab1*$^{-/-}$ mice, as described previously [23]. T cell depletion was performed using anti-CD90–microbeads and an AutoMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

Immunosuppressive Treatment

RAPA was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Everolimus (RAD) and CSA were synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Everolimus emulsion was dissolved in distilled water at a concentration of 625 μg/mL and administered to recipients by oral gavage at a dose of 5 mg/kg. RAPA and CSA were given as suspensions in carboxymethylcellulose sodium salt: CMC (C5013; Sigma-Aldrich, St. Louis, MO) at a final concentration of .2% CMC. RAPA and CSA were administered to recipients by peritoneal injection at doses of .5 and 20 mg/kg, respectively [15,24]. Immunosuppressive treatments were performed once daily, starting on day 0 and continuing until death or end of the observation period (day 110 to 125).

Adoptive Transfer

Splenocytes were isolated from [*H2-Ab1*$^{-/-}$ → C3H] chimeras 6 to 11 weeks after TCD-BMT. $CD4^+$ T cells were negatively selected from splenocytes by depletion of $CD8^+$, $DX5^+$, $CD11b^+$, $Ter\text{-}119^+$, and $B220^+$ cells using the AutoMACS system, as described previously [23]. A total of 2 × 10^7 $CD4^+$ T cells per mouse were injected intravenously into recipients after immunosuppressive therapy for 70 days after BMT.

Assessment of GVHD

After BMT, survival was monitored daily, and weight changes were assessed twice per week. The degree of clinically acute GVHD was assessed twice per week using a scoring system that sums changes in 5 clinical

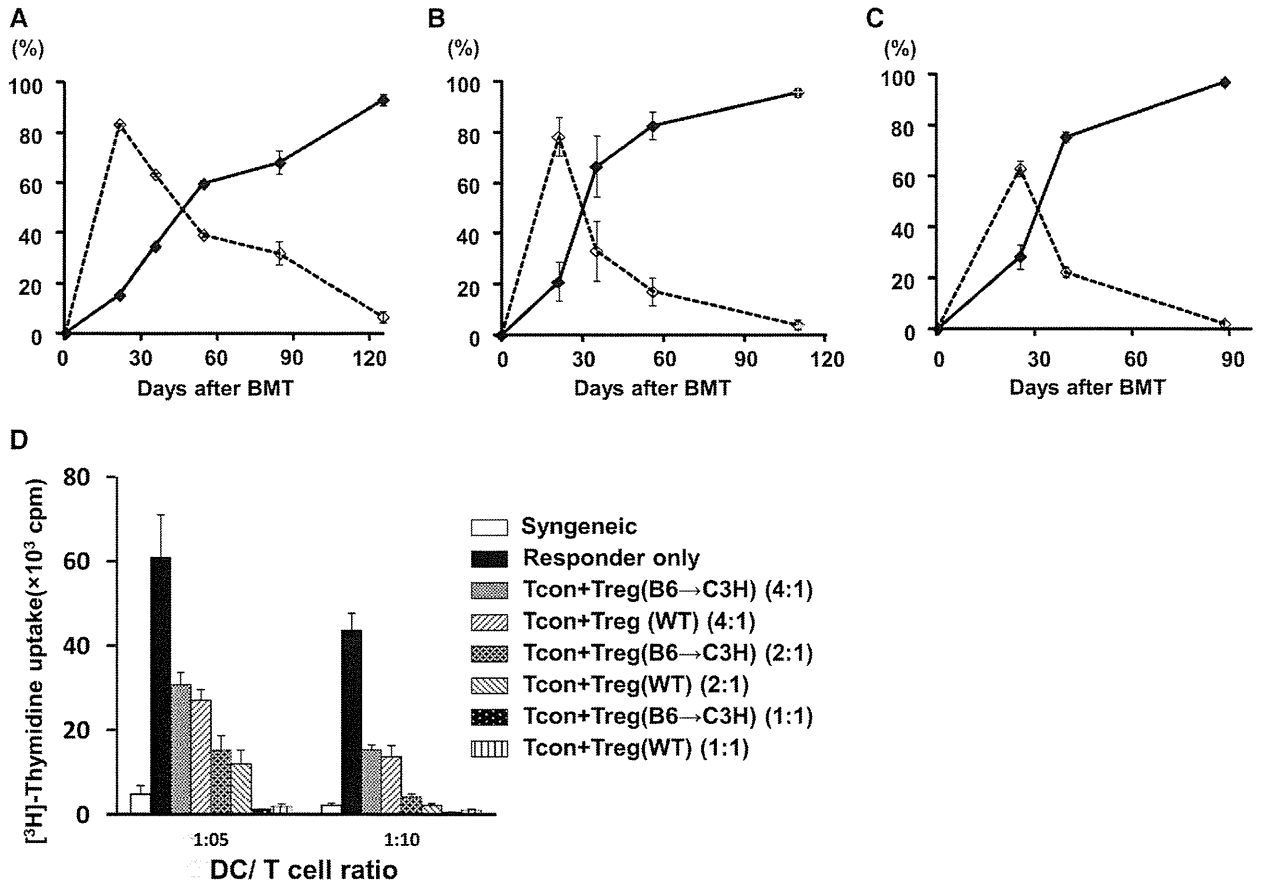

Figure 2. Donor BM–derived progenitors comprise the long-term peripheral Treg pool. Lethally irradiated C3H recipients underwent transplantation as in Figure 1: (B6 → C3H). The rates of $CD45.2^+$ spleen cell–derived (broken lines) and $CD45.2^-$ BM–derived (solid lines) Treg in $CD4^+Foxp3^+$ Treg are shown. Spleen (A) and mesenteric lymph nodes (MLN) (B) were isolated from (B6 → C3H) mice at various time points after BMT and cells were analyzed by fluorescent activated cell sorter. (C) Lethally irradiated C3.SW ($H\text{-}2^b$) recipients underwent transplantation from B6 ($H\text{-}2^b$) donors. The rates of $CD45.2^+$ splenic T cell–derived (broken lines) and $CD45.2^-$ BM–derived (solid lines) Treg in $CD4^+Foxp3^+$ Treg in the spleen are shown. Each group consisted of 20 to 23 mice. The means (±SE) of each group are shown. Data are from a representative of at least 2 independent experiments. (D) $CD25^+CD4^+$ Treg were purified from the spleens of (B6 → C3H) mice (on day 120) or naïve B6 (WT). B6 $CD4^+CD25^-$ T cells (Tcon) together with various numbers of Treg were cultured with irradiated C3H $CD11c^+$ DC as stimulators for 72 hours. Proliferative activities were determined by monitoring ^{3}H-thymidine uptake.

parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10), as described previously [22]. Shaved skin from the interscapular region (approximately 2 cm^2), liver, and salivary gland specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin. Skin slides were scored on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop out (0 to 2 for each category; the maximum score was 10) [21]. Liver slides were scored based on bile duct injury and inflammation (0 to 4 for each category), and the maximum score was 8 [25]. Salivary gland slides were scored based on atrophy and inflammation (0 to 3 for each category), and the maximum score was 6. All slides were scored by pathologists (T.K. and T.T.) blind to experimental group.

Immunohistochemistry

Immunohistochemical staining for Foxp3 and CD3 was performed using the high polymer (HISTOFINE simple stain, NICHIREI, Tokyo, Japan) method. Anti-Foxp3 (eBioscience) and anti-CD3 (Abcam, Cambridge, MA) were used to identify Tregs and effector T cells, respectively.

Flow Cytometry

The mAbs used were unconjugated anti-CD16/32 (2.4G2); FITC-, PE-, PerCP-, or APC-conjugated anti-mouse CD4, CD25, CD45.1, CD45.2, $H\text{-}2^b$, $H\text{-}2^d$ (BD Pharmingen, San Diego, CA); and Foxp3 (eBioscience, San Diego, CA), as described previously [26]. A Foxp3 staining kit (eBioscience) was used for intracellular staining. Cells were analyzed on a FACSAria flow cytometer with FACSDiva software (BD Immunocytometry Systems, San Diego, CA).

Mixed Leukocyte Reaction

$CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD11c^+$ DC were magnetically separated by AutoMACS using microbeads from a $CD4^+CD25^+$ regulatory T cell isolation kit and CD11c microbeads. $CD4^+CD25^-$ T cells (5×10^4 per well) together with various numbers of $CD25^+CD4^+$ T cells (0 to 5×10^4 per well) were cultured with irradiated (30 Gy) $CD11c^+$ DC as stimulators for 72 hours in 96-well round-bottomed plates. Cells were pulsed with ^{3}H-thymidine (1 μCi [.037 MBq] per well) for a further 16 hours [27]. Proliferation was determined using Topcount NXT (Packard Instruments, Meriden, CT).

Statistics

Data are given as means ± SEM. The survival curves were plotted using Kaplan-Meier estimates. Group comparisons of pathology scores were performed using the Mann-Whitney *U* test. Comparative analysis of cell ratios was performed by the unpaired 2-tailed Student *t*-test or Welch's *t*-test. In all analyses, $P < .05$ was taken to indicate statistical significance.

RESULTS

Kinetics of Treg Reconstitution after Allogeneic BMT

We first examined whether Tregs intermixed in the graft persist in the host for long periods post BMT using the MHC-mismatched model of BMT. Lethally irradiated C3H ($H\text{-}2^k$) recipient mice received 10×10^6 TCD-BM cells from B6.Ly-5a ($H\text{-}2^b$,CD45.1) mice with/without 1 to 2×10^6 spleen cells from B6 ($H\text{-}2^b$,CD45.2) mice. All of the recipients of allogeneic C3H TCD-BM cells from B6 mice and syngeneic mice survived and were resistant to induction of GVHD. Although 100% of

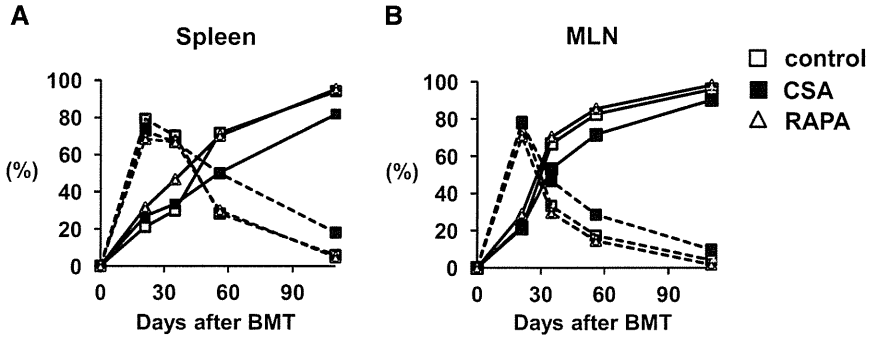

Figure 3. Effects of CSA and mTOR inhibitors on the Treg compartment. Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received i.p. injections of CSA (closed squares), mTOR inhibitor (rapamycin, RAPA; open triangles), or vehicle control (open squares) daily from day 0 to 110. The rates of $CD45.2^+$ splenic T cell–derived (broken lines) and $CD45.2^-$ BM–derived (solid lines) Treg in $CD4^+Foxp3^+$ Treg are shown. Spleen (A) and mesenteric lymph nodes (MLN) (B) were isolated from (B6 → C3H) mice at various time points after BMT and cells were analyzed by fluorescent activated cell sorter. Each group consisted of 16 to 23 mice. The means (±SE) of each group are shown. Data are from a representative of at least 2 independent experiments.

the animals that received allogeneic BM plus 2×10^6 spleen cells died by day 35 with clinical and histopathological signs of severe GVHD, the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed mild clinical signs of GVHD and 60% survived by day 120 (Figure 1A); the following experiment was performed in this setting. Flow cytometric analysis of donor cell chimerism in the spleen 3 weeks after allogeneic BMT showed that 98.8% ± 0.7% of spleen cells were derived from the donor in mice, thus confirming complete donor cell engraftment. Host Tregs, as determined by $CD4^+Foxp3^+H\text{-}2^{k+}$, were not detected in the spleen on day 21 post transplantation (data not shown). On day 21 post transplantation, the majority of $CD4^+Foxp3^+$ Tregs were derived from $CD45.2^+$ splenic T cells (83.4% ± 2.2%), suggesting that splenic T cell–derived Tregs underwent homeostatic and/or alloantigen-driven expansion (Figure 1B) and the absolute number of Tregs in the spleens of the recipients of BM plus Sp cells was significantly higher than in TCD-BM recipients. From day 21 onward, due to GVHD-induced lymphopenia, the absolute number of Tregs in the spleens of recipients of BM plus Sp cells was lower than in TCD-BM recipients (Figure 1C). The rate of $CD45.2^+$ splenic T cell–derived Tregs in $CD4^+Foxp3^+$ Treg decreased gradually and most $CD4^+Foxp3^+$ Treg were $CD45.1^+$ BM-derived (93.2%) on day 125 post transplantation (Figure 2A). The rate of $CD45.1^+$ BM-derived Tregs in the mesenteric lymph nodes (MLN) was also increased and became dominant in the late post-transplantation period (Figure 2B). To exclude strain-dependent artifacts, we next evaluated the kinetics of Treg reconstitution in the B6 ($H\text{-}2^b$) into C3.SW ($H\text{-}2^b$) MHC-compatible, multiple minor histocompatibility antigen (miHA)-incompatible model of SCT. The kinetics of Treg reconstitution in the spleen was similar and most $CD4^+Foxp3^+$ Tregs were derived from $CD45.1^+$ BM (97%) on day 90 post transplantation (Figure 2C). These findings indicated that the peripheral Treg pool was restored first by expanded splenic T cell–derived mature Treg and then by new Tregs generated from donor BM-derived progenitors. Next, to examine the function of newly arising Tregs, purified $CD4^+CD25^+$ T cells on day 120 post transplantation were

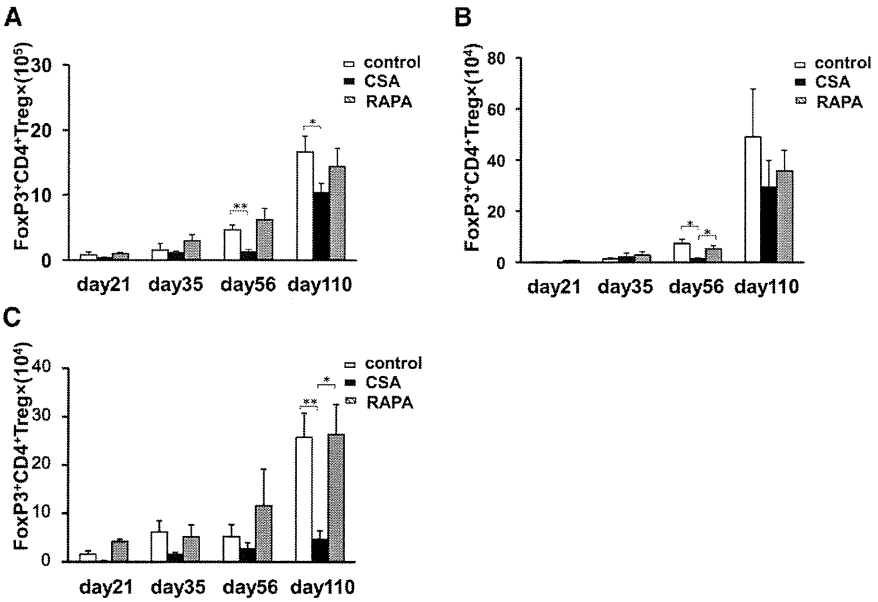

Figure 4. CSA, but not mTOR, inhibitors hampered reconstitution of BM-derived Treg. (B6 → C3H) mice received i.p. injections of CSA (black bars), mTOR inhibitor (rapamycin, RAPA; gray bars), or vehicle control (white bars) daily from day 0 to 110. The absolute numbers of Treg in the spleen (A), MLN (B), and thymus (C) are shown. Each group consisted of 19 to 26 mice. The means (±SE) of each group are shown. Data are from a representative of at least 2 independent experiments. $^*P < .05$; $^{**}P < .01$.

assessed for their ability to inhibit proliferation by responding syngeneic $CD4^+CD25^-$ B6 T cells. Their suppressive activity was virtually indistinguishable from that of Tregs obtained from normal B6 mice (Figure 2D). Taken together, Tregs generated from donor BM-derived progenitors comprise the long-term peripheral Treg pool and exhibit immunosuppressive activity.

CSA, but Not mTORinhibitors, Hampered Reconstitution of BM-derived Treg

Coenen et al. reported that 28 days of CSA administration hampered Treg homeostasis in normal mice [28]. We examined whether the use of CSA for an extended period affected the long-term peripheral Treg pool after BMT. C3H recipient mice underwent transplantation from B6 donor mice (as shown in Figure 1) and received i.p. injection of CSA, mTOR inhibitor (rapamycin; RAPA), or vehicle control daily from day 0. We analyzed the effects of CSA and RAPA on the Treg compartment at 21, 35, 56, and 110 days post hematopoietic cell transplantation. Mice treated with CSA or RAPA showed the same Treg reconstitution pattern as those treated with vehicle solution. On day 21 post transplantation, the majority of $CD4^+Foxp3^+$ Tregs in the spleen were $CD45.2^+$ splenic T cell–derived cells but the Treg compartments were dominated by BM-derived cells on days 56 and 110 post transplantation in all 3 groups (Figure 3A). In the MLN, these 3 groups also showed similar Treg reconstitution kinetics (Figure 3B). There were no differences in the absolute numbers of Treg among the 3 groups on day 21. From day 21 onward, however, the absolute numbers of Tregs in the CSA-treated mice were lower than those in control mice both in the spleen (day 56: $1.3 \pm .4$ versus $4.6 \pm .8 \times 10^5$, $P < .01$; day 110: 10.4 ± 1.4 versus $16.7 \pm 2.4 \times 10^5$, $P < .05$) (Figure 4A) and in the MLN (day 56: $1.3 \pm .5$ versus $7.4 \pm 1.6 \times 10^4$, $P < .03$; day 110: 2.9 ± 1.0 versus $4.9 \pm 1.9 \times 10^5$, $P = .46$) (Figure 4B). Especially in the thymus, mice treated with CSA showed a marked reduction in the absolute numbers of Tregs compared with those treated with vehicle control (day 110: 4.6 ± 1.8 versus $25.7 \pm 5.0 \times 10^4$, $P < .01$) (Figure 4C). In contrast to mice treated with CSA, mice treated with RAPA showed no reduction in the absolute numbers of Tregs and no differences compared with control mice in the spleen or MLN at any time point post transplantation (Figure 4A,B). The absolute numbers of newly arising Tregs in the thymus were also not reduced in mice treated with RAPA (Figure 4C). We next examined the effects of another mTOR inhibitor, everolimus (RAD), which exhibits greater polarity than RAPA and has been approved in Europe for use as an immunosuppressant for prevention of cardiac and renal allograft rejection. Reconstitution of newly arising Tregs in the thymus was not impaired in mice treated with RAD, and there were no differences in the absolute numbers of spleen Tregs compared with control mice on day 110 (spleen: 15.4 ± 2.5 versus $16.6 \pm 2.4 \times 10^5$, $P = .73$, Supplemental Figure 1A; thymus: 17.4 ± 3.2 versus $25.7 \pm 5.0 \times 10^4$, $P = .26$, Supplemental Figure 1B). These findings suggested that CSA, but not mTOR inhibitors, hampered the long-term reconstitution of BM-derived Tregs.

CSA, but Not mTOR Inhibitors, Increased Liability to Chronic GVHD

Recent studies revealed the association of reduced Treg frequency in patients with chronic GVHD. In the present study, we examined histopathological change in CSA-treated mice where reconstitution of BM-derived Tregs was impaired. The skin of CSA-treated mice showed pathogenic features of chronic GVHD (Figure 5A), and pathogenic scores revealed significantly exacerbated chronic GVHD pathology compared with those treated with vehicle control ($5.5 \pm .8$ versus $1.6 \pm .3$, $P < .01$) (Figure 5B). A dry mouth is one of the distinctive features of chronic GVHD. Lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of CSA-treated mice (Figure 5A) and pathological scores were significantly higher in CSA-treated

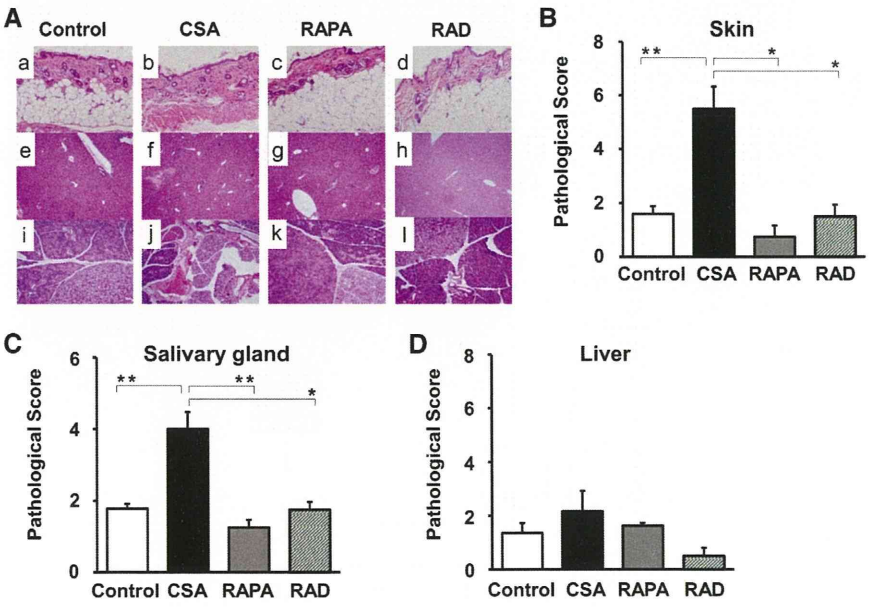

Figure 5. CSA, but not mTOR, inhibitors increased the likelihood of chronic GVHD. (A) Histological findings of the skin (a to d), liver (e to h), and salivary glands (i to l) (on day 120) from (B6 → C3H) mice given CSA, mTOR inhibitor (RAPA, RAD), or vehicle control. Sclerodermatous skin changes, such as epidermal atrophy, fat loss, follicular dropout, and dermal thickness (b); fibrosis in the portal area and peripheral mononuclear cells infiltrates in the liver (f); and fibrosis and atrophy of acinar tissue in the salivary glands (j) were observed (original magnification: × 100) Pathological scores of skin (B), salivary gland (C) and liver (D). The data are expressed as means ± SE. Data are from a representative of at least 2 independent experiments. $^*P < .05$; $^{**}P < .01$.

mice than in the controls (4.0 ± .5 versus 1.8 ± .1, $P < .01$) (Figure 5C). CSA-treated mice showed bile duct injury and fibrosis in the portal area and peripheral mononuclear cell infiltration in the liver and pathological scores of the liver also tended to be worse in CSA-treated mice, as compared with those treated with vehicle control, although it was not statistically significant (Figure 5D). In contrast to mice treated with CSA, mice treated with RAPA showed no pathogenic features of chronic GVHD and there were no differences in pathogenic skin and salivary gland scores, as compared with control mice (skin: .75 ± .4 versus 1.6 ± .3, $P = .18$, Figure 5B; salivary gland: 1.25 ± .2 versus1.78 ± .1, $P = .08$, Figure 5C). Immunohistochemical staining for Foxp3 and CD3 revealed that $CD3^+$ T cells infiltrated in the skin tissue of all 3 groups, and RAD-treated mice showed abundant infiltration by $CD3^+$ T cells and $Foxp3^+$ cells (Figure 6A). In contrast to RAD, $Foxp3^+$ cells were scarcely found in skin tissue of CSA-treated mice. The ratio of Foxp3 Tregs per 100 $CD3^+$ lymphocytes in the skin tissue of CSA-treated mice was significantly lower than those in RAD-treated mice (3.23 ± .4 versus 19.5 ± 4.4, $P < .05$). CSA-treated mice tended to show poorer survival, as compared with those treated with mTOR inhibitors or vehicle control (CSA 27.6% versus control 54.2%, RAD 57.1%, RAPA 61.5%, $P = .28$, Supplemental data Figure 2). These findings suggested that CSA, but not mTOR inhibitors, hampered the reconstitution of BM-derived Treg and increased liability to chronic GVHD.

We next tested liability to chronic GVHD in CSA-treated mice using adoptive transfer experiments. Previously, Sakoda et al. demonstrated that impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD (Figure 7A) [23]. Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient ($H2\text{-}Ab1^{-/-}$) B6 mice ([$H2\text{-}Ab1^{-/-} \rightarrow$C3H]). These mice developed disease conditions that showed all of the clinical and histopathological features of human chronic GVHD. $CD4^+$ T cells isolated from chronic GVHD mice ([$H2\text{-}Ab1^{-/-} \rightarrow$C3H] $CD4^+$ T cells) cause chronic GVHD when B6 antigens are provided by hematopoietic cells in the absence of B6 antigen expression on target epithelium ([B6→C3H] chimeras) [23]. In the current study, C3H mice underwent transplantation from B6 donors as shown in Figure 1 and were orally administered CSA, RAPA, or vehicle solution until 60 days post BMT, when none of the recipients showed significant signs of chronic GVHD. To test liability to chronic GVHD, these C3H-recipient mice with B6-derived antigen presenting cells received adoptive transfer of [$H2\text{-}Ab1^{-/-} \rightarrow$C3H] $CD4^+$ T cells (Figure 7B). As shown in Figure 7C and D, adoptive transfer of pathogenic $CD4^+$ T cells caused severe weight loss (CSA 81.1 ± 4.1% versus control 94.5 ± 2.1%, $P < .05$; and CSA 81.1 ± 4.1% versus RAPA 98.9 ± 1.5%, $P < .01$) and chronic GVHD in CSA-treated mice, with a mortality rate of 83%. RAPA-treated mice and controls showed resistance to induction of chronic GVHD by transfer of pathogenic $CD4^+$ T cells; the

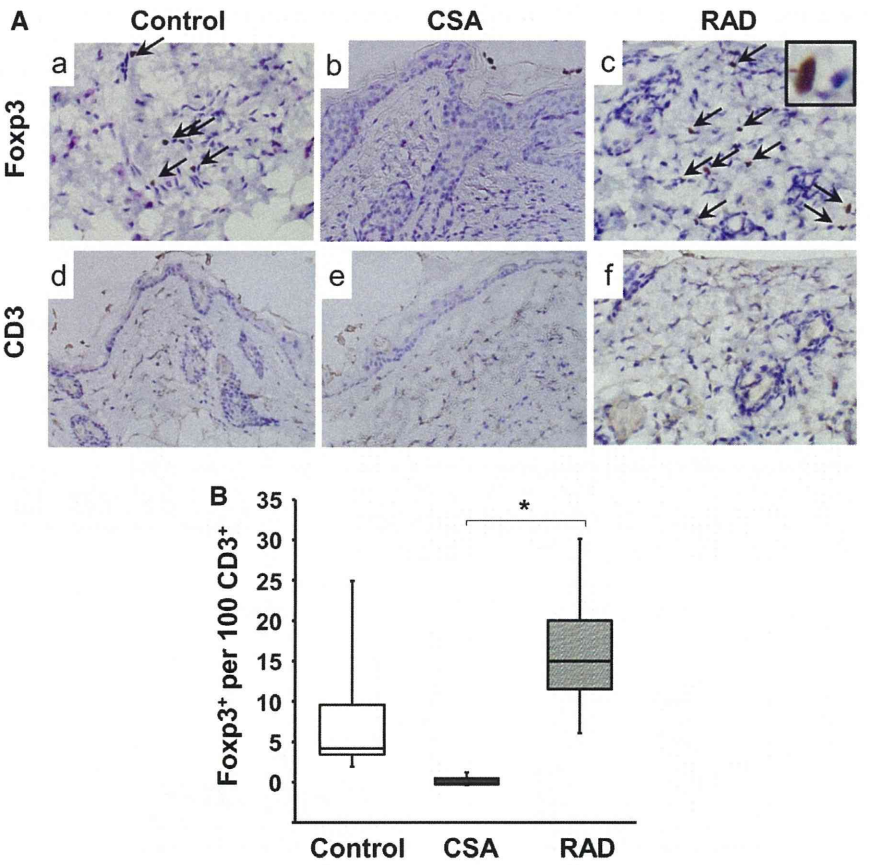

Figure 6. CSA, but not mTOR, reduces Treg infiltration in skin tissue. (A) Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received vehicle control (a, d), CSA (b, e), or mTOR inhibitor (RAD; c, f), daily from day 0 to 120. Immunohistochemical staining was performed using anti-Foxp3 (a to c) and anti-CD3 (d to f) antibodies on day 120. Arrows indicate Foxp3 positive cells. (B) The ratio of Foxp3 Tregs per 100 $CD3^+$ lymphocytes. The number of CD3 and Foxp3 cells was counted in all the high-power fields. Results are expressed as mean ± SD. Pictures and data are from a representative of 2 independent experiments. (n = 3 to 4 per group). *$P < .05$.