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Altered regulatory T cell homeostasis in patients with CD4⁺ lymphopenia following allogeneic hematopoietic stem cell transplantation

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CD4⁺CD25⁺Foxp3⁺ Tregs have an indispensable role in the maintenance of tolerance after allogeneic HSC transplantation (HSCT). Patients with chronic graft-versus-host disease (GVHD) have fewer circulating Tregs, but the mechanisms that lead to this deficiency of Tregs after HSCT are not known. Here, we analyzed reconstitution of Tregs and conventional CD4⁺ T cells (Tcons) in patients who underwent allogeneic HSCT after myeloablative conditioning. Following transplant, thymic generation of naive Tregs was markedly impaired, and reconstituting Tregs had a predominantly activated/memory phenotype. In response to CD4⁺ lymphopenia after HSCT, Tregs underwent higher levels of proliferation than Tcons, but Tregs undergoing homeostatic proliferation also showed increased susceptibility to Fas-mediated apoptosis. Prospective monitoring of CD4⁺ T cell subsets revealed that Tregs rapidly expanded and achieved normal levels by 9 months after HSCT, but Treg levels subsequently declined in patients with prolonged CD4⁺ lymphopenia. This resulted in a relative deficiency of Tregs, which was associated with a high incidence of extensive chronic GVHD. These studies indicate that CD4⁺ lymphopenia is a critical factor in Treg homeostasis and that prolonged imbalance of Treg homeostasis after HSCT can result in loss of tolerance and significant clinical disease manifestations.

Introduction

Allogeneic HSC transplantation (HSCT) provides curative therapy for patients with various hematologic malignancies, bone marrow failure syndromes, and congenital immune deficiencies. With improvements in immune suppressive therapy and supportive care, fewer patients develop acute graft-versus-host disease (GVHD) and more patients survive beyond the first year after transplant. However, the incidence of chronic GVHD has not improved in recent years, and chronic GVHD has become one of the most common and clinically significant problems affecting long-term HSCT survivors (1, 2). Chronic GVHD often presents with clinical manifestations that resemble those of autoimmune diseases such as systemic lupus erythematosus, Sjögren syndrome, and scleroderma (3, 4). Similarly to autoimmune diseases, both T and B cell responses appear to play a role in the pathogenesis of chronic GVHD, suggesting that this reflects a general loss of tolerance including abnormalities in the function of Tregs.

CD4⁺CD25⁺Foxp3⁺ Tregs are a functionally distinct subset of mature T cells with broad suppressive activity (5, 6). Tregs play a key role in the maintenance of peripheral tolerance, and deficiencies of Tregs lead to progressive autoimmune disorders (7, 8). Similarly, enhancement of Treg function can prevent allograft rejection and suppress tumor immunity (9, 10). These observations indicate that an appropriate balance between Tregs and effector T cells is critical

for the maintenance of peripheral tolerance (11). In the setting of allogeneic HSCT, Tregs have also been shown to play an important role in the establishment of tolerance between recipient tissues and donor-derived immunity. This was initially demonstrated in murine studies in which depletion of Tregs from the stem cell graft resulted in increased GVHD and increasing Tregs resulted in suppression of GVHD after transplant (12–15). In humans, we and others previously reported that patients with active chronic GVHD have a lower frequency of Tregs when compared with patients without chronic GVHD (16, 17). These findings suggest that robust reconstitution of Tregs after HSCT is needed to establish a well-balanced immune system that can maintain appropriate levels of peripheral tolerance. However, the mechanisms responsible for reconstitution of Tregs after HSCT have not been well characterized, and the factors that contribute to inadequate recovery of Tregs in patients who develop chronic GVHD are not known.

Following myeloablative conditioning and transplantation of unfractionated HSCs from allogeneic donors, the initial phase of T cell reconstitution is primarily dependent on peripheral expansion of mature T cells that are present in the stem cell graft (18). This is promoted by lymphopenia-related signals as well as stimulation by allo-antigens. Undifferentiated HSCs also migrate to the thymus, where naive T cells with a diverse TCR repertoire are generated and exported into the blood and peripheral lymphoid tissues. Thymus-dependent generation of donor T cells is generally delayed and incomplete in adult patients because of natural thymic involution and damage resulting from high-dose chemotherapy and irradiation adminis-

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1
Patient characteristics: cohort 1

	Patients n = 33	%
Age, median (range)	45 (21 to 57)	
Female	16	49
Disease		
ALL	4	12
AML	18	53
MDS/AML	6	18
CML	3	9
NHL	1	3
Other	1	3
Disease risk status prior to transplant		
Standard risk	22	67
Donor		
MRD	24	72
MUD	9	27
Pretransplant conditioning		
TBI/CY	33	100
Acute GVHD prophylaxis		
MTX/tacrolimus	11	33
Sirolimus/tacrolimus	22	67
Immune suppressive medications at sample collection/d		
Prednisone, 0 mg	19	58
Prednisone, 0 to ≤10 mg	9	27
Prednisone, >10 mg	5	15
Sirolimus, 0 mg	15	45
Sirolimus, 0 to 1 mg	14	42
Sirolimus, >1 mg	4	12
Tacrolimus, 0 mg	11	33
Tacrolimus, 0 to 4 mg	17	52
Tacrolimus, >4 mg	5	15
MMF, 0 mg	25	76
MMF, 1000 to 1500 mg	3	9
MMF, ≥ 2000 mg	5	15
Acute GVHD grade after transplant		
0	23	70
1	4	12
2	5	15
3	1	3
Chronic GVHD status at sample collection		
None	23	70
Inactive	3	9
Limited	4	12
Extensive	3	9
Days from transplant to sample collection, median (range)	272 (75 to 1394)	

NHL, non-Hodgkin lymphoma; MRD, HLA-matched related donor; MUD, HLA-matched unrelated donor; TBI, total body irradiation; CY, cyclophosphamide; MTX, methotrexate; MMF, mycophenolate mofetil.

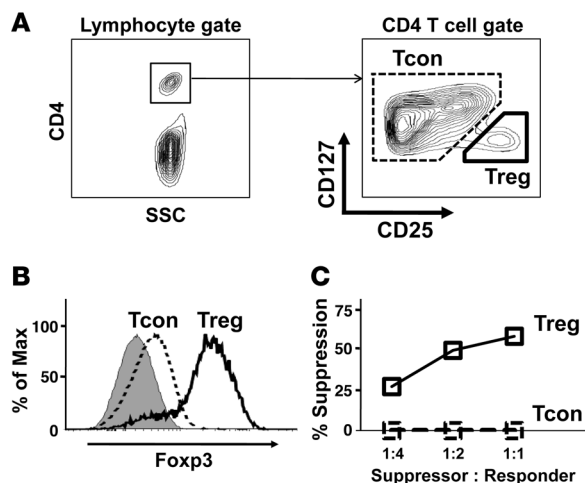
tered as part of the myeloablative regimen (19). Once naive T cells are exported into the periphery, these cells are subject to homeostatic signals that regulate the expansion and contraction of the T cell population to maintain total T cell numbers (and T cell subsets) at appropriate levels in the circulation and peripheral lymphoid tissues.

Recent studies have demonstrated that different T cell subsets are subject to distinct homeostatic controls in vivo (20). In particular, Treg homeostasis appears to be distinct from conventional CD4⁺ T cells (Tcons), and this may contribute to imbalance of Tregs and Tcons in some instances (21, 22). For this reason, our analysis of Treg reconstitution after allogeneic HSCT was designed to directly compare Tregs and Tcons in each patient as well as with normal donors. Our initial studies in a cohort of 33 patients examined primarily in the first year after transplant demonstrated that thymic generation of Tregs was markedly impaired but that this subset maintained a significantly higher level of proliferation compared with Tcons. Treg proliferation in vivo appeared to be driven primarily by CD4⁺ lymphopenia. Importantly, high levels of Treg proliferation were counterbalanced by increased susceptibility to apoptosis in this subset. These studies demonstrate that human Tregs and Tcons respond differently to homeostatic signals and therefore reconstitute in distinct ways after allogeneic HSCT. To examine the clinical relevance of these observations, we prospectively studied an independent cohort of 45 patients after allogeneic HSCT. This analysis confirmed the critical role of CD4⁺ lymphopenia in the recovery of Tregs and demonstrated the selective peripheral depletion of Tregs in response to this prolonged homeostatic pressure. The depletion of peripheral Tregs in these patients was also associated with the development of extensive chronic GVHD.

Results

Phenotypic and functional characterization of CD4⁺CD25^{med-hi}CD127^{lo} Tregs and Tcons after allogeneic HSCT. A detailed analysis of Treg homeostasis following allogeneic HSCT was carried out in 33 adult patients who received myeloablative conditioning (Table 1). These studies focused on the reconstitution of Tregs within the first year after HSCT, and patient samples were obtained at a median of 9 months after transplant. In this analysis, Tregs were always compared with Tcons in the same patient samples. As shown in Figure 1A, Tregs were identified as CD4⁺CD25^{med-hi}CD127^{lo} and Tcons were identified as CD4⁺CD25^{neg-lo}CD127^{med-hi} (23, 24). Tregs identified by these markers in patient samples were confirmed to have high levels of intracellular Foxp3 expression and in vitro suppressive activity (Figure 1, B and C). In contrast, CD4⁺ Tcons had little expression of Foxp3 and no suppressive activity in vitro.

Thymic generation and peripheral proliferation of Tregs after allogeneic HSCT. T cell reconstitution following allogeneic HSCT can occur through 2 distinct pathways. One pathway is thymic-dependent generation of T cells from donor hematopoietic progenitor cells, and the other is through peripheral expansion of mature T cells that are also present in the donor stem cell product (18). To determine the relative contribution of each pathway to the reconstitution of Tregs and Tcons, we compared the presence of recent thymic emigrants (RTEs) and proliferating cells in each subset in every patient sample. CD31 (PECAM-1) has recently been demonstrated to be an excellent marker of RTEs in specific T cell populations including Foxp3⁺ Tregs (25–28). In our analysis, RTEs were measured by the coexpression of CD45RA and CD31 in both Treg and Tcon subsets (Figure 2A). Cell proliferation in each subset was measured by quantifying the expression of Ki-67 (Figure 2A). Ki-67 is a critical protein for cell division and is expressed exclusively by proliferating cells (29). As shown for a representative patient in Figure 2A, the Tcon population contained both naive (CD45RA⁺) and memory cells (CD45RA⁻). The majority of naive Tcons expressed CD31, but very few of these cells expressed Ki-67, indicating a low level of

**Figure 1**

Expression of Foxp3 and suppressive activity of CD4⁺CD25^{hi}CD127^{lo} Tregs after allogeneic HSCT. (A) Representative lymphocyte gate for identification of CD4⁺ T cell subsets is shown. Within the CD4⁺ T cell gate, Tregs are identified as CD25^{med-hi}CD127^{lo} and Tcons are identified as CD25^{neg-lo}CD127^{med-hi}. (B) Gated Tregs and Tcons were examined for intracellular Foxp3 expression. Representative data are shown. Shaded histogram represents isotype control. (C) Tregs or Tcons isolated from patient peripheral blood were cultured with responder Tcons from the same patient and stimulated with irradiated allogeneic PBMCs for 5 days. Method for calculating percentage suppression of proliferation is described in Methods. Data are representative of 5 independent experiments.

proliferation within this population. In contrast, the Treg population contained very few naive CD45RA⁺CD31⁺ cells, but proliferating cells expressing Ki-67 were readily detected.

Results of this analysis for all patients in cohort 1 are summarized in Figure 2B. In healthy donors, Tregs had significantly fewer RTEs and a higher fraction of proliferating cells compared with Tcons (22). After allogeneic HSCT, the frequency of RTEs was significantly reduced in both Treg and Tcon subsets. In particular, very few RTEs were detected in the Treg subset after HSCT. In contrast, cell proliferation after HSCT was significantly increased in both Tcon and Treg subsets compared with healthy donors. This was most evident for Tregs after HSCT, which showed significantly increased Ki-67 expression compared with Tregs in healthy donors and Tcons after HSCT. To further characterize the specific Treg subset containing actively proliferating cells after HSCT, we examined Ki-67 expression within each Treg subset by flow cytometry (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI41072DS1). Both CD31⁺CD45RA⁺ and CD31⁺CD45RA⁺ memory Treg subsets contained a relatively high proportion of Ki-67-expressing cells. In contrast, the CD31⁺CD45RA⁺ RTE Treg subset contained very few proliferating cells.

Patient samples were obtained at various times during the first year after transplant. This allowed us to examine the relationship between levels of RTEs and proliferating cells and the time after HSCT in each subset (Figure 2C). In Tcons, the frequency of RTEs gradually increased during the first year as thymic function recovered after transplant. However, cell proliferation within the Tcon subset remained stable and at relatively low levels throughout this period. In contrast, RTEs in Tregs remained at very low levels during the entire first year after HSCT. Cell proliferation remained high in Tregs throughout this entire period. Taken together, these results indicate that Treg reconstitution during the first year after HSCT occurs primarily through active proliferation rather than through thymic generation of naive Tregs.

To identify factors that induce active proliferation of Tregs after HSCT, we examined the relationship between the level of Treg proliferation and specific laboratory and clinical variables at the time of sample collection. As shown in Figure 2D, the level of Treg proliferation was inversely correlated with CD4⁺ T cell count of the patient. We also found higher levels of Treg proliferation in patients who received rapamycin (sirolimus) for acute GVHD prophylaxis compared with patients who received other regimens

from multivariable linear models (Table 2). There was no significant correlation with other clinical factors, including patient age, disease status, acute GVHD, chronic GVHD, and time after transplant (Table 2). These results suggest that active proliferation of Tregs is primarily driven by CD4⁺ lymphopenia, and the use of sirolimus may enhance this effect.

Tregs exhibit activated/memory proapoptotic phenotype after HSCT. Experiments in mice have suggested that cells undergoing lymphopenia-induced proliferation (LIP) develop an activated/memory phenotype and become more susceptible to apoptosis in vivo (30–33). To determine whether this effect influenced Treg and Tcon reconstitution in humans after HSCT, we first examined the expression of several established cell differentiation/activation markers on Tregs and Tcons in patient samples. Results in patients were compared with those of healthy adult donors and umbilical cord blood samples (Table 3). As expected, both Tcons and Tregs from cord blood had a predominantly CD45RA⁺ naive phenotype, and Tcons and Tregs from healthy adult donors contained fewer CD45RA⁺ naive cells (34). After HSCT, both Tregs and Tcons exhibited higher levels of activation markers, including CD39 and HLA-DR, compared with healthy donors, but this was more evident in Tregs than Tcons. Because the expression of CD39 and HLA-DR on Tregs is restricted to the functional effector/memory subset (35, 36), these results provide further evidence that Treg reconstitution is characterized by a marked shift in the activated/memory status of these cells (37). As shown in Table 3, Tregs do not express CD69, a well-established early activation antigen after transplantation. Although CD69 expression on Tregs has not previously been examined, experiments in mice have shown that CD69 is not expressed by CD8⁺ T cells undergoing LIP (30, 31), while this marker is expressed on CD4⁺ T cells and CD8⁺ T cells after alloantigen stimulation in vivo (38). Thus, the absence of CD69 expression on Tregs suggests that lymphopenia-related mechanisms play a more important role than antigen stimulation in supporting proliferation of Tregs after HSCT.

CD95 (FAS) expression was examined in greater detail, since it is known that induction of apoptosis through this pathway plays an important role in limiting the expansion of proliferating T cells in vivo (39–41). Few cord blood Tcons expressed CD95, but 60%–65% of Tcons from healthy donors and patient samples expressed this marker (Table 3). Tregs from all 3 sources had consistently higher numbers of CD95⁺ cells than the corresponding Tcons (Table 3), and expression of this marker was significantly greater in Tregs after HSCT than in healthy donors ($P < 0.0001$). As shown in Figure 3A, after HSCT, Tregs were almost entirely CD45RA⁺ and expressed high levels of CD95. Tcons in the same samples were also

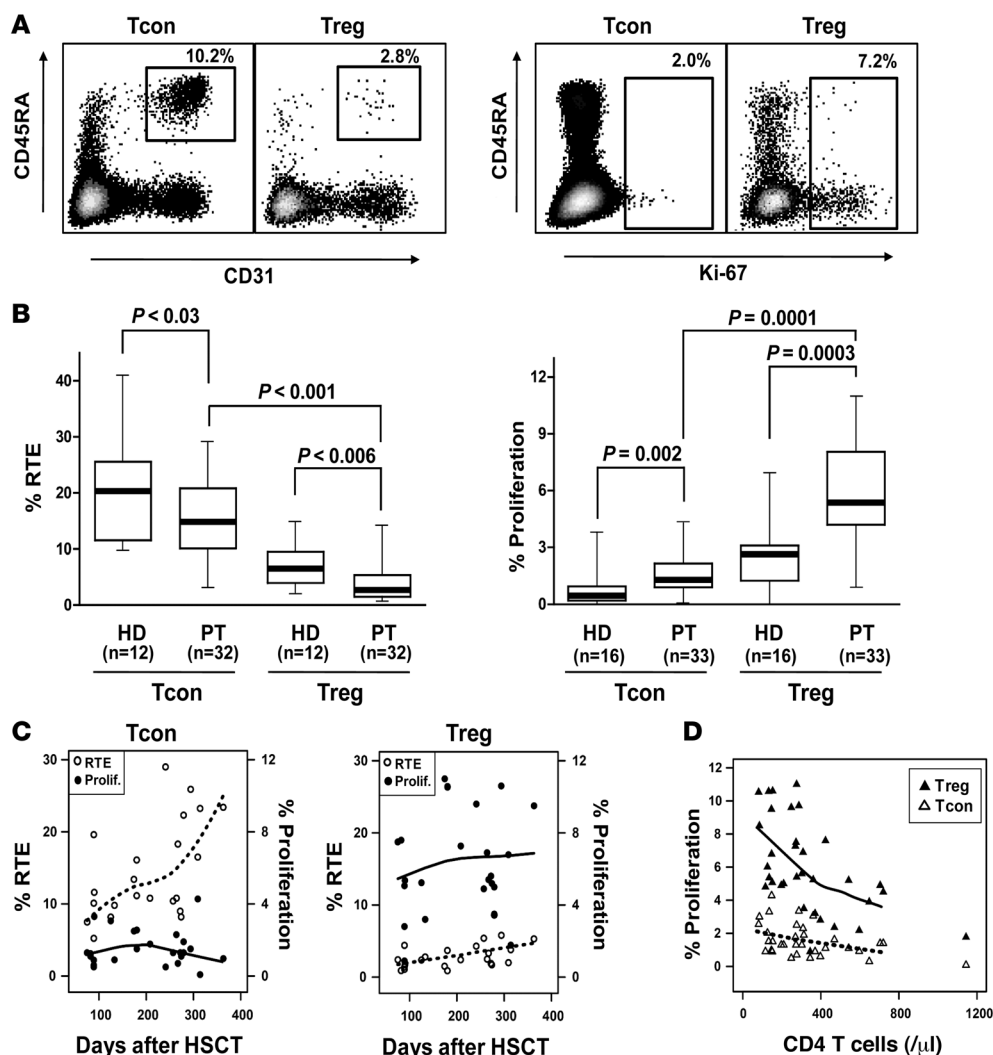


Figure 2

Reduced thymic generation and increased peripheral proliferation of Tregs after allogeneic HSCT. Gated Tregs and Tcons were examined for CD31 and Ki-67 expression to determine the percentage of RTEs and proliferating cells in each subset. **(A)** Representative panels for identification of RTEs (left) and proliferating cells (right) are shown. RTEs are identified as CD45RA⁺CD31⁺ cells, and proliferating cells are identified as Ki-67⁺ cells. **(B)** The percentages of RTEs (left) and proliferating cells (right) in each CD4⁺ subset from post-HSCT patients (PT) and healthy donors (HD) are shown. Box plots in each figure depict the 75th percentile; median and 25th percentile values and whiskers represent maximum and minimum values. **(C)** Levels of RTEs (open circles, left y axis) and proliferating cells (closed circles, right y axis) are shown at different times in the first year after HSCT. Results are shown separately for Tcons (left panel) and Tregs (right panel). **(D)** Correlation of percentage of Ki-67⁺ proliferating cells in each CD4⁺ T cell subset and peripheral blood CD4⁺ T cell count (μ l) of the patient are shown. Tregs (closed triangles) are compared with Tcons (open triangles). Tregs: $r = -0.56$, $P = 0.0008$; Tcons: $r = -0.32$, $P = 0.08$; Spearman's test.

biased to a CD45RA⁺ memory phenotype but still retained a subset of CD45RA⁺CD95⁺ naive T cells. The patient example shown in Figure 3A also indicated that the level of CD95 expression in individual cells was greater in Tregs than in Tcons. After HSCT, the level of CD95 expression in CD45RA⁺ Tregs measured by MFI was inversely correlated with the CD4⁺ lymphocyte count in the sample. No similar correlation was found for CD95 expression in CD45RA⁺ Tcons (Figure 3B). To determine whether the expression of CD95 was directly related to proliferation, we compared MFI for CD95 in CD45RA⁺ Tregs and CD45RA⁺ Tcons categorized by either high or low levels of Ki-67⁺ cells. The median value of percentage of Ki-67 in each subset was used as a cut-off value for this categorization. The expression of CD95 was significantly greater in high-

proliferating Tregs (HP; %Ki-67⁺ $\geq 5.0\%$) than low-proliferating Tregs (LP; %Ki-67⁺ $< 5.0\%$). In contrast, there was no difference in CD95 expression in Tcon samples with either high (%Ki-67⁺ $\geq 1.0\%$) or low levels of proliferation (%Ki-67⁺ $< 1.0\%$) (Figure 3C).

BCL-2 expression in Tregs and Tcons during reconstitution after allogeneic HSCT might influence the survival of circulating cells (41, 42). It has previously been reported that after HSCT, T cells have lower levels of BCL-2 expression than healthy donor T cells (43). To examine this issue, we categorized Tregs and Tcons into "naive" and "activated/memory" subsets by the expression of CD45RA and examined BCL-2 expression in each of these 4 subsets. This demonstrated that CD45RA⁺ Tregs had significantly lower expression of BCL-2 than the other 3 subsets (Figure 4A).

**Table 2**Clinical factors associated with expression of Ki-67 in T cell subsets^A

Confounding factors	% Ki-67 in Tcons P value ^B	% Ki-67 in Tregs P value ^B
Age (≥50 vs. <50)	0.53	0.50
Sex (F vs. M)	0.83	0.14
Risk status (CR1 vs. no CR1)	0.94	0.48
Tacrolimus/sirolimus vs. tacrolimus/MTX	0.21	0.017
Acute GVHD grade(2–4 vs. 0–1)	0.45	0.07
Chronic GVHD (No vs. Yes)	0.54	0.80
Sample date after HSCT	0.95	0.27

^AData are obtained from 33 patients in cohort 1. ^BP values from multi-variable linear models. CR1, first complete remission.

Reduced BCL-2 expression in CD45RA⁺ Tregs was associated with the number of proliferating cells (%Ki-67) in this subset (Figure 4B), suggesting that BCL-2 expression in Tregs is reduced during the conversion from naive to memory phenotype in LIP in vivo. Collectively, these results demonstrate that patient Tregs selectively exhibit an apoptosis-prone phenotype and suggest that this is driven by persistent lymphopenia following HSCT.

Tregs exhibit increased susceptibility to TCR- and Fas-mediated apoptosis in vitro after HSCT. To determine whether the apoptosis-prone phenotype of Tregs actually leads to increased susceptibility to apoptosis, we carried out in vitro functional assays with purified cells. First, 4 different CD4⁺ T subsets were purified by cell sorting from patient blood (Figure 5, A and B) and cells were labeled with CFSE. Then each subset was cultured in the presence of anti-CD3 and anti-CD28 antibodies and IL-2 for 4 days. Harvested cells were stained with anti-CD4 and anti-Foxp3 monoclonal antibodies and analyzed by flow cytometry. Alive and apoptotic cells were estimated by side versus forward scatter profile (Figure 5C). As shown in Figure 5C, fewer CD45RA⁺ Tregs survived in comparison with the 3 other subsets. Naive CD45RA⁺ Tregs showed the highest levels of both proliferation and upregulation of Foxp3 expression (44). In contrast, only a small fraction of CD45RA⁺ Tregs survived, and few

of these cells showed evidence of proliferation. Moreover, Foxp3 expression was also lost after several divisions (Figure 5C). These results indicate that memory Tregs from HSCT patients are highly susceptible to apoptosis and have a limited proliferative capacity after TCR stimulation in vitro.

To examine mechanisms of apoptosis in Tregs, we measured CD95-induced apoptosis in each of these CD4⁺ populations after isolation by cell sorting. Apoptosis was detected by costaining with annexin V and 7-amino-actinomycin D (7-AAD) at 3, 6, and 18 hours after apoptosis induction, and the effects of anti-Fas antibody were compared with control cells cultured in medium alone. In healthy donors, CD45RA⁺ Tregs were the only subset that contained apoptotic cells, and there was a relatively small increase in the level of apoptosis during the 18-hour examination period (Figure 6, A and B) (45). After HSCT, CD45RA⁺ Tregs showed high levels of apoptosis that increased dramatically during the observation period. All other CD4⁺ T cell subsets after HSCT remained resistant to apoptosis (Figure 6, A and B). Of note, the degree of apoptosis in CD45RA⁺ Tregs was inversely correlated with both CD4⁺ T lymphocyte count (Figure 6C) and the proportion of CD45RA⁺ naive subset to total CD4⁺ T cells (Figure 6D). Since CD4⁺ T cells convert from naive to memory phenotype during lymphopenic proliferation, these correlations again suggest that lymphopenia-driven proliferation underlies the Fas-mediated apoptosis of CD45RA⁺ Tregs.

Long-term Treg reconstitution is impaired in patients with prolonged severe CD4⁺ lymphopenia. The comparison of phenotypic and functional characteristics of patient Tregs and Tcons suggested that CD4⁺ lymphopenia preferentially affects Treg homeostasis, as these cells reconstitute after allogeneic HSCT. To assess the impact of CD4⁺ lymphopenia on the reconstitution of Tregs after HSCT, we prospectively monitored lymphocyte reconstitution in an independent cohort of 45 patients who had undergone allogeneic HSCT from HLA-identical donors after myeloablative conditioning (Table 4). To evaluate the effect of CD4⁺ T lymphopenia on the reconstitution of CD4⁺ T cell subsets, we categorized the patients into 2 groups based on the CD4⁺ T cell count that was observed 6 months after HSCT. 24 patients whose CD4⁺ T cells were greater than or equal to 260/μl were categorized as group A and 21 patients whose CD4⁺ T cells were less than 260/μl at this time were categorized as group B. Total CD4⁺ T cell reconstitu-

Table 3

Expression of cell-differentiation and activation markers in T cell subsets

CD4 ⁺ subset	Surface marker	Cord blood		Healthy Donor		After HSCT		P value HD vs. HSCT
		n	Median (range)	n	Median (range)	n	Median (range)	
Tcons	CD39	5	0.5 (0.3 to 0.6)	8	1.6 (1.2 to 3.6)	9	3.9 (1 to 5.5)	0.03
	CD45RA	5	97.5 (97.2 to 98.6)	12	39.0 (18.6 to 62.9)	15	29.0 (9.4 to 56.9)	0.2
	CD69	3	0.1 (0.1 to 0.4)	5	0.2 (0.1 to 0.6)	5	0.2 (0.1 to 0.5)	>0.99
	CD95	4	3.8 (2.2 to 9.5)	10	59.9 (37.6 to 76.5)	17	65.1 (45 to 90.8)	0.44
	HLA-DR	4	0.6 (0.2 to 1)	11	3.7 (2 to 7.8)	26	8 (1.6 to 35.8)	0.003
Tregs	CD39	5	9.8 (4.2 to 13.4)	8	29.7 (11.2 to 52.1)	9	49.9 (30.2 to 79.7)	0.04
	CD45RA	5	86.5 (85.1 to 88.2)	12	19 (5.4 to 35.5)	15	6.1 (1.9 to 19.9)	0.002
	CD69	3	0.1 (0 to 0.5)	5	0.9 (0 to 2.1)	5	1.3 (0.5 to 1.7)	>0.99
	CD95	4	36.6 (26.5 to 38.4)	10	79.6 (70.4 to 89.3)	17	94.9 (83.2 to 99.5)	<0.0001
	HLA-DR	4	2.4 (0.9 to 3.7)	11	31.2 (21 to 46.7)	26	48.9 (20.1 to 73.6)	0.01

Values represent median percentage of cells expressing corresponding markers within gated Tcon or Treg subsets obtained from cord blood, healthy adult blood, and post-HSCT patients.

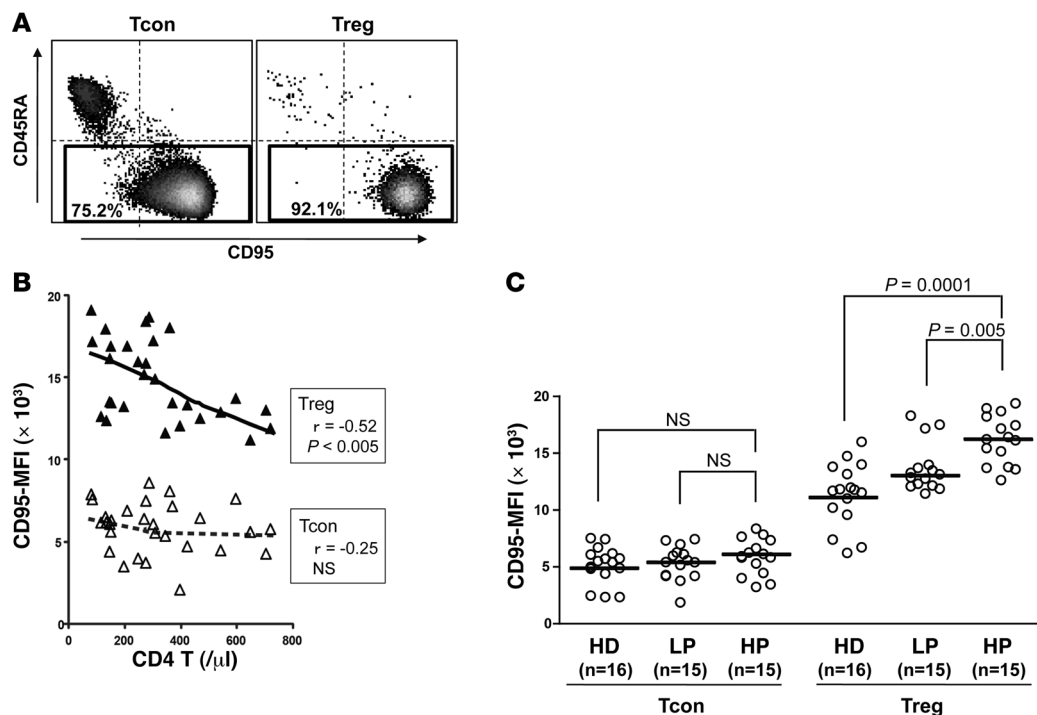


Figure 3

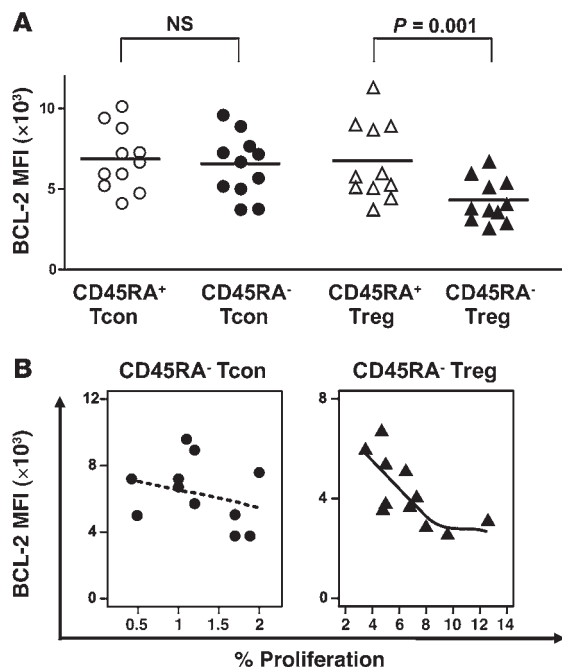
Increased expression of Fas (CD95) on CD45RA⁺ Tregs is associated with low CD4⁺ T cell count. Gated Tregs and Tcons were examined for expression of Fas (CD95). **(A)** Representative flow cytometry profiles from post-HSCT patients are shown. CD45RA⁺ populations in each subset are gated for determination of CD95 expression. **(B)** Correlation between MFI of CD95 staining on CD45RA⁺ Tcons (open triangles) or Tregs (closed triangles) and peripheral blood CD4⁺ T cell count (cells/ μL) for each patient sample are shown. Correlation between the 2 variables was calculated using a rank-based Spearman's test (Tregs: $r = -0.52$, $P < 0.005$; Tcons: $r = -0.25$, $P = 0.19$). **(C)** MFI of CD95 staining is shown for both CD45RA⁺ Tcon and Treg subsets from each patient sample. Within each subset, results are compared for samples with high levels of proliferating cells (HP) and low levels of proliferating cells (LP) based on the median percentage of Ki-67 in each sample. The expression of CD95 was significantly greater in high-proliferating cells (%Ki-67⁺ $\geq 5.0\%$) than in low-proliferating cells (%Ki-67⁺ $< 5.0\%$). In contrast, there was no difference in CD95 expression in Tcon samples with either high (%Ki-67⁺ $\geq 1.0\%$) or low levels of proliferation (%Ki-67⁺ $< 1.0\%$). Results are also compared with values in healthy donors.

tion in these 2 groups is shown in Figure 7A. In group A, CD4⁺ T cell counts gradually improved over the 2-year period of observation and achieved normal levels (>800 cells/ μL) by 18 months after transplantation. Patients in group B remained severely lymphopenic during this entire period, and CD4⁺ T cell counts remained less than 400 cells/ μL until 2 years after transplantation. Figure 7B compares the recovery of CD4⁺ Tregs and CD4⁺ Tcons in each group. To compare the patterns of recovery in the same graph, different scales were used in these panels (see Figure 7 legend). In group A, both Tcons and Tregs increased gradually during the 2-year period of observation. In group B, Tcons increased gradually over time, even though the absolute counts were consistently about 50% less than those in group A. In group B, Tregs increased in the first 9 months after HSCT, and the absolute levels achieved were similar to those of group A during this period. However, with persistent lymphopenia, Treg levels subsequently declined and remained at very low levels from 12 to 24 months after HSCT.

To evaluate the impact of Treg reconstitution patterns on clinical outcome, we determined the incidence and severity of chronic GVHD in each group (Figure 7C). In patients with relatively well-balanced recovery of CD4⁺ T cell subsets (group A), 29% developed extensive chronic GVHD and 29% never developed chronic GVHD. In contrast, 77% of patients with persistent CD4⁺ lymphopenia and

impaired reconstitution of Tregs (group B) developed extensive chronic GVHD, and only one patient (5%) never developed chronic GVHD. We further assessed the timing of onset of chronic GVHD in each group. Since the initial symptoms of chronic GVHD are often vague, we utilized the time to initiation of steroid therapy (prednisone dose ≥ 30 mg/day) as an indicator of onset of clinically significant extensive chronic GVHD (Figure 7D). In the first 9 months after HSCT, Treg numbers were similar in group A and group B and the incidence of chronic GVHD was not significantly different during this period ($P = 0.29$). Subsequently, the incidence of extensive chronic GVHD plateaued in group A, as both Tcons and Tregs continued to recover. In contrast, the incidence of extensive chronic GVHD continued to increase in group B, as Tcon numbers slowly increased and Treg numbers declined. This resulted in a significantly higher incidence of extensive chronic GVHD in group B compared with group A at 2 years after transplant ($P = 0.002$). These results suggest that abnormal Treg homeostasis can affect the induction and maintenance of peripheral tolerance following HSCT and demonstrate the clinical significance of abnormal reconstitution of Tregs in this setting.

Altered plasma cytokine levels after allogeneic HSCT. The common γ chain cytokines are known to play important roles in T cell homeostasis. Maintenance of CD4⁺ Tcons and CD8⁺ T cells are dependent

**Figure 4**

Reduced expression of BCL-2 in CD45RA⁻ Tregs undergoing proliferation. Gated Tregs and Tcons were further subdivided into naive and activated/memory subsets based on the expression of CD45RA. Subsequently, each subset (CD45RA⁺ Tcons, CD45RA⁻ Tcons, CD45RA⁺ Tregs, and CD45RA⁻ Tregs) was examined for intracellular BCL-2 expression. (A) MFI of BCL-2 staining is shown in each subset. Black bars depict median values. (B) Reduced expression of BCL-2 is significantly correlated with percentage of Ki-67 in CD45RA⁻ Tregs (right) but not with CD45RA⁻ Tcons (left). Correlation between the 2 variables was calculated using a rank-based Spearman's test (Tregs: $r = -0.77$, $P < 0.01$; Tcons: $r = -0.18$, $P = 0.59$).

on signals mediated by IL-7 and IL-15 (46), whereas CD4⁺ Treg homeostasis is maintained by IL-2 (47, 48). To examine the role of these cytokines in CD4⁺ T cell homeostasis after allogeneic HSCT, we measured levels of IL-2, IL-7, and IL-15 in plasma in cohort 2 patients at defined time points after transplant. As shown in Figure 8A, plasma IL-2 levels were not elevated in the first year after HSCT and were similar to the low levels present in healthy donors. Plasma IL-7 and IL-15 levels were higher than those in healthy donors at all times after transplant. Each cytokine level was compared with CD4⁺ T cell numbers at 6, 9, and 12 months after transplant (Figure 8B). Levels of IL-2 and IL-7 did not correlate with CD4⁺ T cell count, but increased levels of IL-15 were significantly associated with CD4⁺ lymphopenia. The relationship of each cytokine to Tcon and Treg counts was also examined (Table 5). IL-15 levels were significantly associated with low numbers of CD4⁺ Tcons, but only in the first year after HSCT. High IL-7 levels persisted for at least 2 years after HSCT, but were not associated with reconstitution of CD4⁺ Tcons. No significant correlation was observed between Treg number and any of the 3 cytokines, including IL-2. Finally, levels of IL-2, IL-7, and IL-15 were compared in groups A and B (data not shown). IL-2 and IL-7 levels were similar in groups A and B. IL-15 levels were significantly higher in group B at 6, 9, and 12 months after transplant, likely reflecting the lower levels of Tcons in these samples.

Discussion

Patients with active chronic GVHD who are at more than 2 years after transplantation are known to have a deficiency of Tregs (16). However, the factors that lead to the relative deficiency of Tregs in these patients were not known. We examined the process of Treg reconstitution in the first year after transplant with the goal of identifying abnormalities that might lead to the persistent deficiency of Tregs in these patients. Since the reconstitution of Tregs following transplantation occurs in a profoundly lymphopenic environment, we focused on factors known to play a role in T cell homeostasis. In

this analysis, Tregs were compared with Tcons in the same patient samples as well as with Tregs and Tcons in healthy donors. This allowed us to identify distinct responses of Tregs and Tcons to the same homeostatic signals in vivo and to document changes that occurred at various times during reconstitution of donor T cell immunity after allogeneic HSCT. To undertake this study, we focused on patients who received myeloablative conditioning, and therefore T cell reconstitution is derived entirely from the donor stem cell graft. All hematopoietic grafts were provided by HLA-matched healthy donors and were obtained by apheresis after stem cell mobilization with G-CSF. Two independent groups of patients were studied, and the cellular composition of the infused stem cell products was similar in both cohort 1 and 2 (Supplemental Table 1). All patients received prophylactic immune suppressive agents after transplant, but none of the patients received anti-thymocyte globulin and none of the stem cell products were modified to deplete T cells from the stem cell graft. These individuals therefore represented a large and relatively uniform population of patients in which we could examine reconstitution of normal donor T cell populations after allogeneic stem cell transplantation.

One major conclusion from this analysis is that CD4⁺ Tcons and CD4⁺ Tregs respond very differently to homeostatic signals, as these populations reconstitute in the first year after HSCT. After HSCT, Tcons have higher numbers of proliferating cells (Ki-67⁺) than healthy donors and also have a predominantly memory phenotype (CD45RA⁻). However, as thymic function recovers, increasing numbers of RTEs (CD45RA⁺CD31⁺) appear within the Tcon subset. In contrast, reconstituting Tregs contain very few RTEs, even after thymic function has recovered and when naive cells are readily apparent within the Tcon subset. Instead, the Treg population contains a relatively high proportion of proliferating cells that is maintained throughout the first year after HSCT. The Treg population in healthy donors also contains fewer naive cells and greater numbers of proliferating cells than the normal Tcon population, but these differences are significantly exaggerated during lymphocyte reconstitution after transplantation. We observed that CD4⁺ lymphopenia selectively induces Treg proliferation after HSCT. Thus, Treg homeostasis appears to be modulated by the level of recovery of Tcons.

Further analysis of Treg subsets revealed that Ki-67-expressing cells were found primarily within CD45RA⁻ memory Treg populations and not in the CD45RA⁺CD31⁺ RTE subset. This observation may suggest that activated/memory Tregs can expand to compensate for the reduced thymic output of Tregs after HSCT. Alternatively, RTE Tregs may rapidly convert to a CD45RA⁻ activated/memory phenotype when exposed to severely lymphopenic conditions in the periphery. Recently, Miyara et al. demonstrated

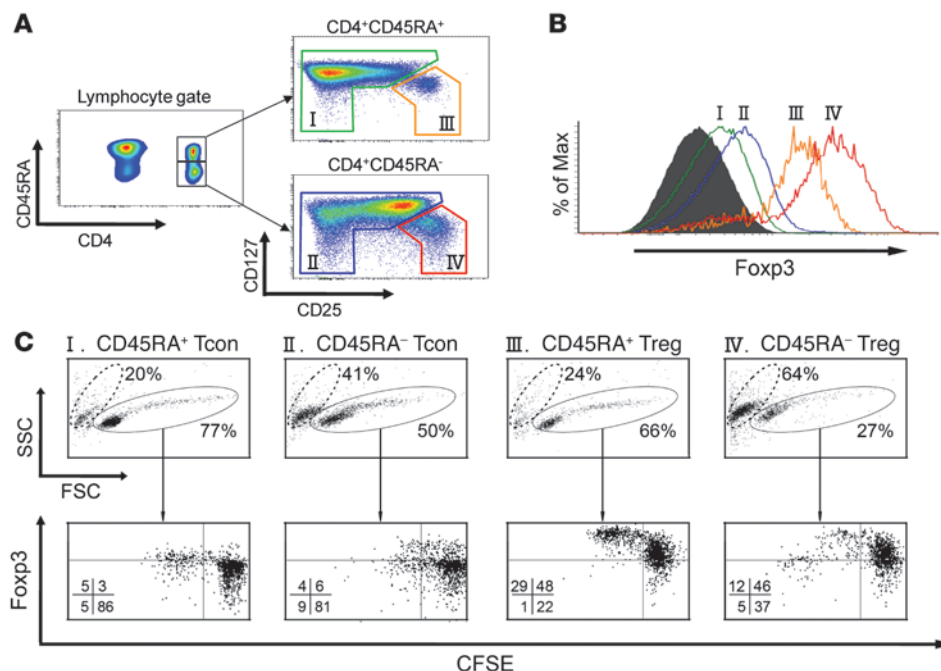


Figure 5

Increased susceptibility to apoptosis and limited proliferative potential in memory Tregs after allogeneic HSCT. **(A)** Gating procedure for isolation of CD45RA⁺ naive Tcons (gated with green), CD45RA⁻ memory Tcons (gated with blue), CD45RA⁺ naive Tregs (gated with orange), and CD45RA⁻ memory Tregs (gated with red) by cell sorting. Representative patient sample is shown. **(B)** Expression of Foxp3 was determined for each CD4⁺ T cell subset. The results of each subset are shown with respective colors as shown in **A**. A representative patient sample is shown. Shaded histogram represents isotype control. **(C)** CFSE-labeled cells were cultured in the presence of anti-CD3, anti-CD28, and IL-2. Cells were harvested after 4 days and stained with anti-CD4 and anti-Foxp3. Gated CD4⁺ cells were examined. "Alive" cell gate (solid line) and "apoptotic" cell gate (broken line) were defined on the basis of side versus forward scatter (upper panels). Cells in "alive" cell gate were further examined for CFSE dilution and Foxp3 expression (lower panels). A representative result from 5 independent experiments is shown. Numbers denote percentages of cells in the respective quadrants.

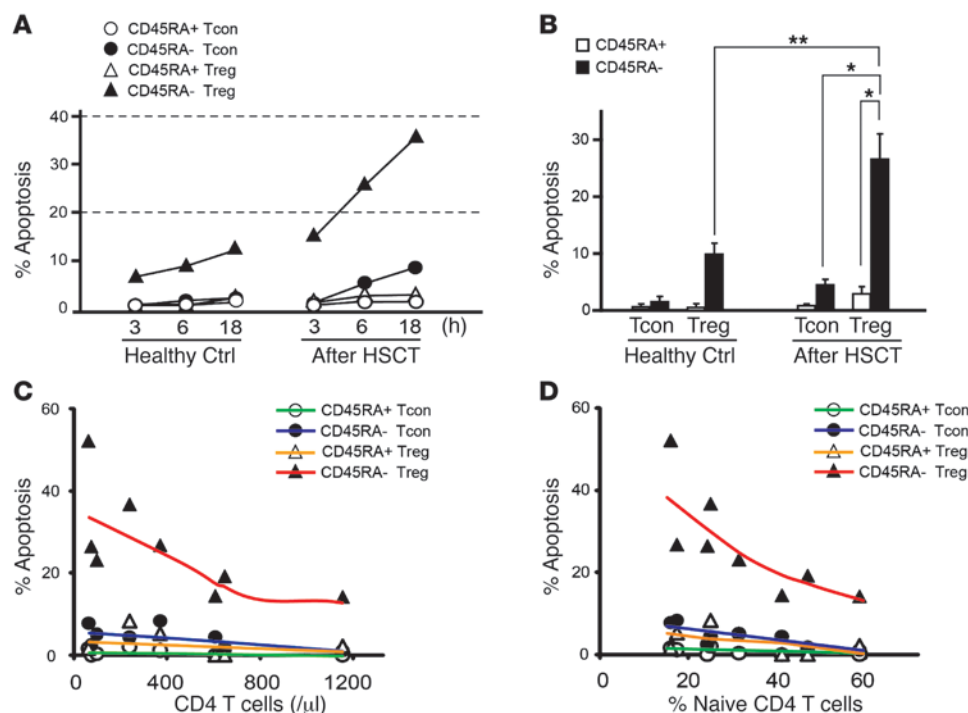
that human Tregs can differentiate from a CD45RA⁺CD31⁺ to a CD45RA⁻ phenotype and that CD45RA⁻ activated Tregs can accumulate in aged donors and in some pathologic settings (37). Our analysis of CD4⁺ T cell reconstitution in HSCT patients indicates that lymphopenic conditions can profoundly affect Treg differentiation and homeostasis and shift overall the balance of Tregs toward an activated/memory population.

Despite maintaining high levels of proliferation, Treg reconstitution is generally delayed after allogeneic HSCT. When compared with Tcons, Tregs express higher levels of Fas (CD95) and lower levels of BCL-2. This proapoptotic phenotype is significantly correlated with both the proportion of proliferating cells in the Treg population and the level of CD4⁺ lymphopenia in the patient after transplantation. Functional studies *in vitro* with purified cells confirmed that this proapoptotic phenotype was associated with significantly increased susceptibility to apoptosis. Fas-induced apoptosis was significantly correlated with the level of CD4⁺ lymphopenia in the patient. Importantly, increased susceptibility to apoptosis was a unique feature of CD45RA⁻ memory Tregs and was not a feature of naive Treg or Tcon populations. Since almost all Tregs after HSCT have an activated/memory phenotype, this helps explain why high levels of Treg proliferation do not result in rapid functional recovery of this important regulatory subset.

Taken together, these studies identified significant differences between reconstitution of Tcons and Tregs after transplantation and suggested a mechanism that could result in exhaustion of

the Treg pool and relative deficiency of Tregs, especially in those patients with persistent CD4⁺ lymphopenia. To validate these findings and examine potential clinical outcomes, we prospectively studied immune reconstitution in an independent cohort of 45 patients undergoing allogeneic HSCT. Because persistent CD4⁺ lymphopenia was identified as a critical factor in our initial studies, the second cohort was divided into 2 groups (Table 4) based on the absolute CD4⁺ T cell levels at 6 months after transplantation. Group A consisted of 24 patients whose CD4⁺ T cells were greater than or equal to 260/ μ l, and group B consisted of 21 patients whose CD4⁺ T cells were less than 260/ μ l. There were no significant clinical characteristics that distinguished these 2 groups at 6 months after transplantation, including the number of patients that had developed acute GVHD. Group A patients had significantly higher total lymphocyte counts as well as CD4⁺ T cell counts. Total Treg counts were slightly higher in group A at 6 months after HSCT, but this difference was not statistically significant.

During a 2-year period of observation following transplantation, total CD4⁺ T cell counts gradually recovered in both groups (Figure 7A). However, T cell recovery was slower in group B, in which median CD4⁺ counts remained below 400 cells/ μ l until 2 years after transplantation. In the first 9 months after transplantation, recovery of Tregs in both groups was quantitatively similar and achieved levels seen in normal individuals (35 cells/ μ l). In this early recovery period, the relative expansion of Tregs compared with Tcons was actually higher in group B. However, after

**Figure 6**

Rapid induction of apoptosis in memory Tregs by anti-Fas and inverse correlation with CD4⁺ T cell count after HSCT. CD45RA⁺ Tcons, CD45RA⁻ Tcons, CD45RA⁺ Tregs, and CD45RA⁻ Tregs were isolated by cell sorting and cultured separately with anti-Fas antibody or control medium. Apoptosis was assessed by annexin V/7-AAD costaining at several time points, and Fas-specific cell death was calculated as described in Methods. **(A)** Time-dependent percentage of Fas-specific apoptosis is shown for each CD4⁺ T cell subset. Data are representative of results from 8 post-HSCT patients and 4 healthy donors. **(B)** Results of percentage of Fas-specific apoptosis 6 hours after induction are summarized (mean + SEM). * $P < 0.01$, ** $P = 0.02$. **(C and D)** Correlation between percentage of Fas-specific apoptosis in each CD4⁺ subset after 6 hours and peripheral blood CD4⁺ T cell count (cells/ μ l) **(C)** or percentage of CD45RA⁺ naive population of total CD4⁺ T cells **(D)** is shown. Trend lines are shown in respective colors. Correlation between the 2 variables was calculated using a rank-based Spearman's test. Percentage of Fas-specific apoptosis of memory Tregs was inversely correlated with both CD4⁺ T cell count and percentage of naive CD4⁺ T cells (vs. CD4⁺ T cell counts; $r = -0.79$, $P = 0.02$; vs. percentage of naive /CD4⁺ T cells, $r = -0.90$, $P = 0.002$).

9 months, Treg recovery collapsed in group B, and these patients were not able to maintain normal numbers of Tregs in peripheral blood. Tcon recovery was also delayed in group B, but this population continued to increase gradually over time, even as Tregs declined (Figure 7B). These data suggest that even though the lymphopenic environment influences both Tcon and Treg reconstitution, the Treg population is affected differently by this environment. The inability to reconstitute Tregs in group B was associated with a markedly higher incidence of chronic GVHD. In group A, 71% of patients developed either no chronic GVHD or only limited chronic GVHD and only 29% developed extensive chronic GVHD. In contrast, only 23% of patients in group B developed no or limited chronic GVHD and 77% developed extensive chronic GVHD. These differences between group A and B were confirmed when we examined the time of onset of extensive chronic GVHD. Relatively few patients developed extensive chronic GVHD in the first 9 months after HSCT, and the incidence was similar in both groups during this period. Subsequently, Treg numbers declined in group B, and this was associated with a significantly higher incidence of extensive chronic GVHD in this group at 2 years after transplant ($P = 0.002$). These differences in the time of onset as well as the overall incidence of chronic GVHD support our hypothesis that inadequate reconstitution of CD4⁺ Tregs contributes to the development of clinically significant

chronic GVHD. Persistent CD4⁺ lymphopenia appears to play a critical role in this pathologic process, at least in part because this has an important effect on Treg homeostasis.

Although our studies focused on reconstitution of CD4⁺ T cells, we also examined CD8⁺ T cell recovery, as these cells are known to be important cellular mediators of GVHD (Supplemental Figure 2). In group A, the recovery of CD8⁺ T cells generally paralleled the recovery of CD4⁺ Tcons and achieved normal levels (400 cells/ μ l) by 12 months after transplant. Once normal levels were achieved in group A, total CD8⁺ T cell numbers remained stable in the second year after transplant. In group B, CD8⁺ T cells gradually increased in the first year after transplant and also plateaued in the second year, even though normal levels had not been achieved. Although CD8⁺ T cell counts did not decline in group B, these results suggest that persistent lymphopenia may also adversely affect the recovery of CD8⁺ T cells. Further detailed studies of CD8⁺ T cell homeostasis are needed to address this issue.

Our studies demonstrate that Tregs and Tcons have distinct responses to the same lymphopenic environment, but the signaling pathways responsible for these differences are not known. Recent studies have suggested that IL-7 and IL-15 are the primary homeostatic cytokines that drive CD4⁺ Tcon proliferation (46, 49–53). Our analysis found that plasma IL-7 levels are consistently high after allogeneic HSCT, but IL-7 levels do not correlate with



Table 4
Patient characteristics: cohort 2

	Group A (n = 24)		Group B (n = 21)		P value
	n	%	n	%	
Age, median (range)	42 (18 to 58)		42 (22 to 53)		0.67
Female	13	54	9	43	0.55
Disease					0.7
ALL	1	4			
AML	6	25	6	29	
MDS/AML	4	17	4	19	
CML	6	25	8	38	
NHL	6	25	2	10	
Other	1	4	1	5	
Disease risk status prior to transplant					
Standard risk	13	54	13	62	0.76
Donor					
MRD	16	67	17	81	0.33
MUD	8	33	4	19	
Pretransplant conditioning					
TBI/CY					
Acute GVHD prophylaxis					
Sirolimus/tacrolimus	24	100	21	100	
Acute GVHD grade after transplant					0.93
0	13	54	10	48	
1	7	29	8	38	
2	1	4	1	5	
3	3	13	2	10	
Chronic GVHD after transplant					0.006
None	7	29	1	5	
Limited	10	42	4	19	
Extensive	7	29	16	76	
Lymphocyte count 6 months after transplant, median (range)					
Total lymphocytes	1303 (630 to 2744)		646 (270 to 1800)		<0.0001
Total CD4 ⁺ T cells	462 (268 to 997)		176 (41 to 255)		<0.0001
Total CD4 ⁺ Tregs	34 (0 to 518)		19 (4 to 89)		0.18

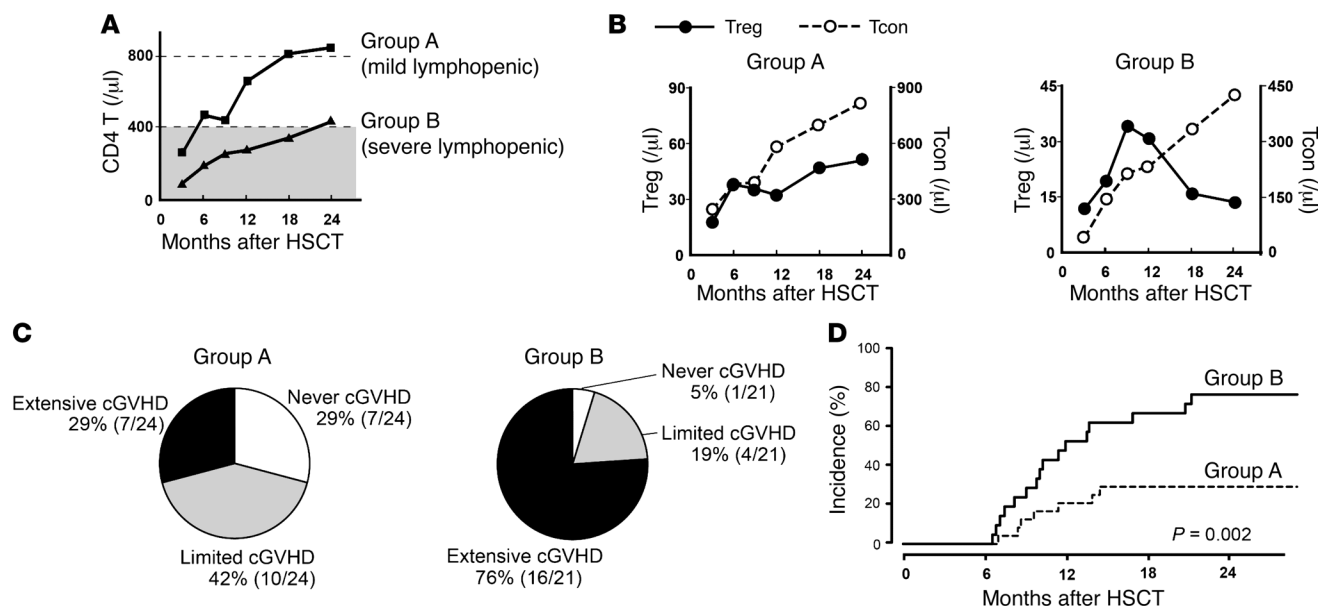
the degree of CD4⁺ T cell recovery. Thus, IL-7 may promote T cell expansion after transplant, but high plasma levels of IL-7 persist after lymphopenia has resolved. IL-15 levels are also elevated after allogeneic HSCT, but in contrast to IL-7, IL-15 levels are increased primarily in patients with severe CD4⁺ lymphopenia. Thus, IL-15 appears to function as a true homeostatic cytokine for CD4⁺ Tcons after transplant. Neither IL-7 nor IL-15 levels were found to be associated with the level of CD4⁺ Treg recovery after transplant, and these cytokines do not appear to play a direct role in CD4⁺ Treg homeostasis in this setting. However, through influencing CD4⁺ Tcon reconstitution, IL-15 may nevertheless indirectly modulate CD4⁺ Treg recovery. It is also important to consider that plasma cytokine levels may not accurately reflect the activity of these cytokines in the microenvironments where they exert important local immunoregulatory functions. Thus, further studies are needed to precisely define the potential roles of IL-7 and IL-15 in Treg reconstitution and homeostasis after transplantation.

IL-2 is known to be a critical homeostatic cytokine for Tregs in vivo (47, 48, 54–57), but plasma levels of IL-2 remain low after allogeneic HSCT and were not correlated with Treg recovery.

Previous murine experiments have suggested that IL-2 is not required for LIP of Tregs (48), and this is consistent with our studies demonstrating high levels of Treg proliferation after transplant without concomitant increased plasma levels of IL-2. Notably, all patients in our study received calcineurin inhibitors for prophylaxis of acute GVHD after transplant. These agents inhibit the ability of activated T cells to produce IL-2 and likely contribute to a relative IL-2 deficiency as Tregs reconstitute after transplant. Although plasma IL-2 levels do not change as peripheral Treg numbers recover, IL-2 levels are also low in healthy individuals and plasma levels may not accurately reflect the concentrations of IL-2 in the microenvironments in which activated T cells secrete IL-2 and modulate Treg expansion. Thus, the lack of correlation between plasma IL-2 levels and Treg reconstitution may not exclude an important role for IL-2 in the reconstitution of CD4⁺ Tregs after HSCT. Moreover, cell-intrinsic mechanisms are also likely to play important roles in controlling Treg proliferation as well as the susceptibility of these cells to apoptosis after activation (58, 59). Further studies to identify both extrinsic and intrinsic factors that regulate the homeostatic proliferation of Tregs are clearly needed. Clarification of these cellular mechanisms will allow the development of new strategies for modulating Treg reconstitution after transplantation and potentially for developing new ways of preventing or treating chronic GVHD.

In the current study, we identified mechanisms of abnormal Treg homeostasis after stem cell transplantation that appear to contribute to a quantitative deficiency of circulating Tregs. In addition, abnormal homeostasis may also lead to functional defects of Tregs in vivo. First, the

limited generation of naive Tregs in the thymus combined with high levels of proliferation and apoptosis may severely limit the TCR repertoire diversity of this population. For example, Treg clones with higher avidity for minor histocompatibility alloantigens are more likely to preferentially expand and then undergo apoptosis under the conditions of immune reconstitution found in our patients. This process may further skew the diversity of the Treg TCR repertoire by selectively depleting those Tregs that are capable of suppressing alloimmune responses in vivo (60, 61). Second, previous studies have shown that the expression of tissue homing receptors on Treg changes during the conversion from naive to activated/memory phenotype (62). Following stem cell transplantation, this may result in a critical decrease of naive Tregs that are able to migrate to secondary lymphoid organs and may further contribute to the inability to suppress the initiation of donor effector T cell responses that occurs in these sites (63–65). Finally, activated/memory Tregs that reside within GVHD target tissues may be more highly susceptible to apoptosis than circulating Tregs because these inflammatory sites are rich in antigenic stimuli including constitutive expression of

**Figure 7**

Impaired reconstitution of Tregs and increased incidence of chronic GVHD in patients with prolonged severe CD4⁺ T lymphopenia. The reconstitution of peripheral blood lymphocyte subsets was assessed prospectively by flow cytometry at 3, 6, 9, 12, 18, and 24 months after HSCT. Median counts (cells/μl) of each subset measured at each time point are shown. **(A)** Patients were categorized into 2 groups by the absolute number of CD4⁺ T cells at 6 months after HSCT. 24 patients with CD4⁺ T cell counts greater than or equal to 260/μl were included in group A and 21 patients with CD4⁺ T cell counts of less than 260/μl were included in group B. Median CD4⁺ lymphocyte counts in the each group at each time point are shown. **(B)** Median Treg counts (solid line, left y axis) and Tcon counts (dashed line, right y axis) in group A and group B patients are shown at serial times over a 2-year period after allogeneic HSCT. Note the scale for Tregs shown on left y axis is one-tenth of the scale for Tcons shown in right y axis. The scales of both left and right y axes for group B are half of the scales shown for group A. **(C)** Incidence and severity of chronic GVHD are compared for group A (left) and group B (right) patients. **(D)** Cumulative incidence of extensive chronic GVHD in group A (broken line) and group B (solid line). *P* value between the 2 groups was calculated using a log-rank test. *P* = 0.29 at 9 months; *P* = 0.002 at 24 months.

alloantigens. Considering these potential defects in Treg function that can occur as a direct result of the abnormalities in Treg homeostasis, it is possible that the deficit in Treg function after allogeneic HSCT may be even greater than the numerical deficit we identified in this study. Importantly, these functional defects may selectively target those Treg populations that most actively regulate responses to allogeneic target antigens and therefore play a critical role in the immune pathology of GVHD.

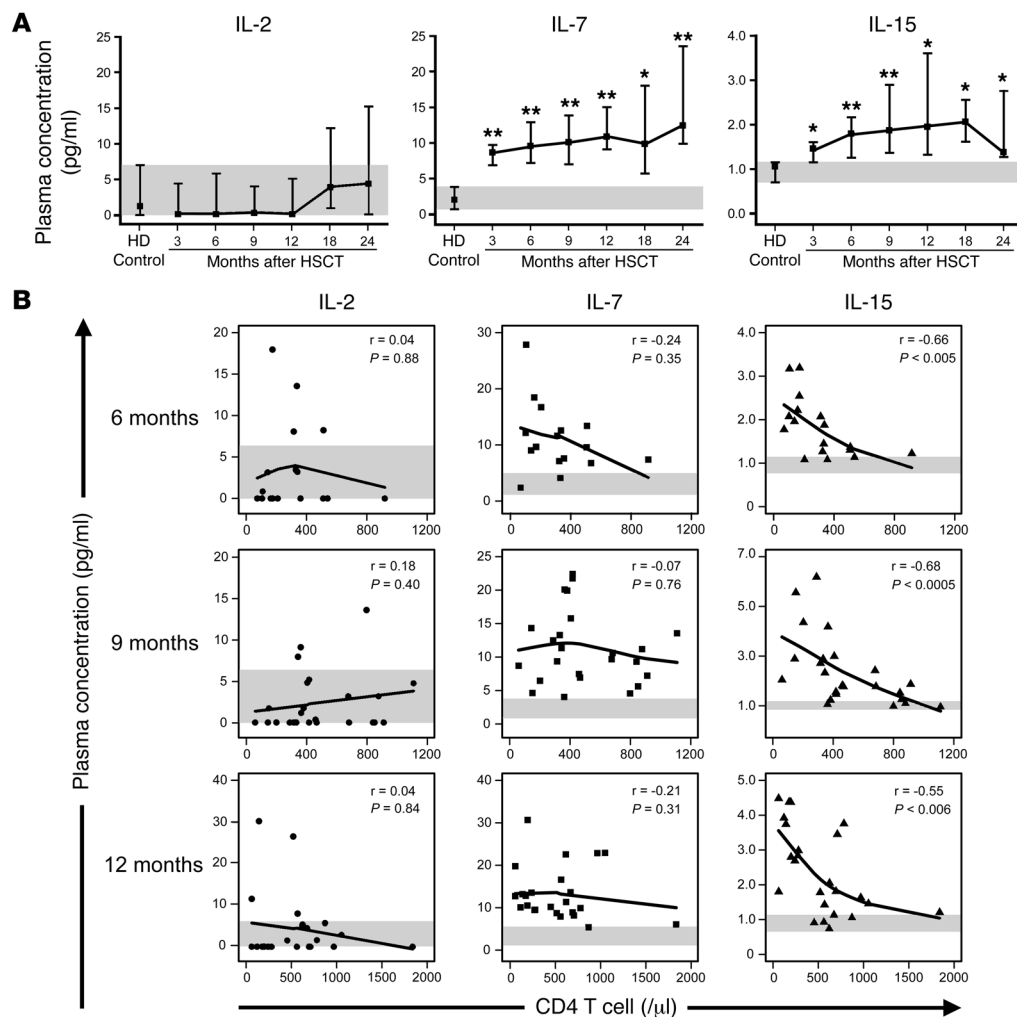
Our studies focused entirely on human allogeneic stem cell transplantation as a model system for studying Treg homeostasis. Autoimmune diseases and treatment-related autoimmune syndromes are also often associated with lymphopenia (66–71). In patients with cancer, infusions of autologous T cells specific for self-tumor-associated antigens appear to be more effective when administered to patients with lymphopenia (72, 73). Recent studies in mouse models and patients with autoimmune disease have suggested that lymphopenia-induced expansion of autoreactive T cell clones can be an important factor leading to autoimmunity (51, 74, 75). However, it is not known whether the expansion of potentially autoreactive T cells is itself sufficient to induce autoimmunity under lymphopenic conditions or whether other mechanisms are also required. (76, 77). Tregs play an important role in suppressing preexisting autoreactive T cells and also control the LIP of other T cell subsets to prevent the generation of autoreactive T cell clones (78, 79). Our results indicate that Tregs have a more robust proliferative response to lymphopenia than Tcons, and this may be a general mechanism

that suppresses the generation of autoreactive T cells under these conditions. However, persistent lymphopenia can subsequently result in exhaustion of the Treg pool, potentially leading to loss of self tolerance and the emergence of autoreactive T cells. Chronic GVHD is well known to have features of autoimmunity as well as alloimmunity, and the abnormal homeostatic response of Tregs to a lymphopenic environment may provide a common pathologic mechanism for chronic GVHD and other autoimmune diseases. Considering the broad clinical relevance of this mechanism, the restoration of normal Treg homeostasis should be taken into consideration in the development of new strategies for treatment of these diseases and for promoting restoration tolerance in patients with autoimmunity as well as GVHD.

Methods

Patient characteristics

Laboratory studies described in this report were undertaken in 78 adult patients who underwent allogeneic HSCT at the Dana-Farber Cancer Institute and Brigham and Women's Hospital (Boston, Massachusetts, USA). All patients were enrolled in clinical research protocols approved by the Human Subjects Protection Committee of the Dana-Farber/Harvard Cancer Center. Written informed consent was obtained from each patient prior to sample collection, in accordance with the Declaration of Helsinki. Clinical characteristics of these patients are summarized in Tables 1 and 4. All patients received myeloablative conditioning regimens and standard immunosuppressive regimens for GVHD

**Figure 8**

Altered cytokine milieu during reconstitution of Tregs and Tcons after allogeneic HSCT. Homeostatic cytokines in plasma were measured at 3, 6, 9, 12, 18, and 24 months after HSCT. **(A)** Median values (pg/ml) with interquartile range of each cytokine measured at each time point are shown. Gray belt in each graph depicts the interquartile range of healthy controls. IL-7 and IL-15 levels at each time point were significantly higher in patients than in healthy donor controls. * $P < 0.003$, ** $P < 0.0001$. IL-2 levels in patients were not elevated in the first year. **(B)** Correlation of plasma cytokine concentration and peripheral blood CD4⁺ T cell counts (/μl) are shown at 6, 9, and 12 months after transplant. Only IL-15 levels were significantly correlated with CD4⁺ lymphopenia (Spearman's test).

prophylaxis. No patients received T cell-depleted stem cell products or prophylactic therapy with anti-thymocyte immunoglobulin. All patients underwent transplantation as therapy for hematologic malignancies, and all had received conventional chemotherapy prior to transplantation. Patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) in first complete remission, chronic myelogenous leukemia (CML) in chronic phase, and myelodysplastic syndrome (MDS) with refractory anemia (RA) or RA with ringed sideroblasts (RARS) were characterized as standard risk; all other patients were considered to have high-risk disease. No patients had previously undergone autologous or allogeneic stem cell transplantation. All donors were HLA matched with recipients. Median ages of donors for cohort 1 and cohort 2 patients were 40 and 41 years, respectively. In all cases, donor stem cells were harvested from filgrastim-mobilized peripheral blood and no significant differences were observed in the cellular composition of the grafts between cohort 1 and cohort 2 (Supplemental Table 1). Chronic GVHD

status shown in Tables 1 and 4 was categorized according to documented clinical examinations and laboratory studies using both Seattle and NIH chronic GVHD consensus criteria.

Patient cohort 1. Detailed analysis of CD4⁺ T cell homeostasis following allogeneic HSCT was undertaken in 33 patients. Clinical characteristics of these patients are summarized in Table 1. Blood samples were collected at relatively early times after transplantation (median: 9.0 months). Chronic GVHD status shown in Table 1 was determined at the time of sample collection. We also studied 16 age-matched healthy adults and 5 umbilical cord blood samples.

Patient cohort 2. Longitudinal reconstitution of lymphocyte subsets was prospectively monitored in 45 patients undergoing allogeneic HSCT (Table 4). Samples in this cohort were obtained at 3, 6, 9, 12, 18, and 24 months after HSCT. The number of samples analyzed at each time point were 38, 45, 40, 39, 28, and 21, respectively. Patients undergoing allogeneic HSCT who died within 2 years after HSCT because of treatment-related



Table 5
Relationship of cytokine levels to CD4⁺ T cell subsets

CD4 ⁺ subset	Months after HSCT	IL-2		IL-7		IL-15	
		<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Tcons	6	0.00	0.99	−0.30	0.24	−0.70	0.002
	9	0.16	0.47	−0.22	0.32	−0.62	0.002
	12	0.03	0.88	−0.27	0.20	−0.54	0.007
	18	−0.30	0.34	−0.13	0.70	0.06	0.86
	24	−0.13	0.64	0.18	0.52	−0.34	0.21
Tregs	6	0.29	0.25	0.33	0.20	0.00	0.99
	9	0.07	0.74	0.02	0.91	−0.39	0.06
	12	0.00	0.99	−0.06	0.77	−0.24	0.25
	18	−0.05	0.88	−0.25	0.43	0.03	0.91
	24	−0.01	0.96	0.33	0.23	−0.43	0.11

Correlation between cytokine levels in plasma (pg/ml) and CD4⁺ T cell subset count (cells/ μ l) at each time point was calculated using a rank-based Spearman's test.

toxicity or disease relapse were excluded from this analysis. Patients who developed chronic GVHD before 6 months after HSCT were also excluded. Patients in cohort 2 were divided into 2 groups based on the median absolute CD4⁺ lymphocyte count in peripheral blood 6 months after HSCT. Group A patients had 260 or more CD4⁺ lymphocytes/ μ l and group B patients had less than 260 CD4⁺ lymphocytes/ μ l. Chronic GVHD status shown in Table 4 was maximum status during the course of each patient.

Flow cytometry

For patient cohort 1, PBMCs were isolated from blood samples by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare) and cryopreserved in aliquots before being analyzed. After thawing, PBMCs were first incubated with the following directly conjugated monoclonal antibodies for 20 minutes at 4°C: anti-CD4-Pacific blue (clone RPA-T4; BD Biosciences), anti-CD25-PC7 (clone M-A251; BD Biosciences), anti-CD45RA-FITC (clone M-A251; Beckman Coulter), and anti-CD127-APC-Alexa Fluor 750 (clone eBioRDR5; eBioscience). Cells were washed twice and incubated with the following PE-conjugated monoclonal antibodies in separate tubes: anti-CD31 (clone WM59; BD Biosciences), anti-CD39 (clone TÛ66; BD Biosciences), anti-CD69 (clone FN50; eBioscience), anti-CD95 (clone DX2; BD Biosciences), and anti-HLA-DR (clone LN3; eBioscience). To detect intracellular Foxp3, surface-stained PBMCs were processed using fixation buffer and permeabilization buffer (eBioscience) and incubated with APC-conjugated Foxp3 (clone PCH101; eBioscience). To detect intracellular Ki-67 and BCL-2, surface-stained PBMCs were immediately pelleted in 80% cold ethanol and left at −20°C overnight. Ethanol-fixed PBMCs were washed twice in PBS and incubated with PE-conjugated anti-Ki-67 antibody (clone B56; BD Biosciences) or PE-conjugated anti-BCL-2 antibody (clone Bcl-2/100; BD Biosciences) for 30 minutes at room temperature. CD4⁺ Tcons were defined as CD4⁺CD25^{neg-lo}CD127^{med-hi}, and CD4⁺ Tregs were defined as CD4⁺CD25^{med-hi}CD127^{lo}, as shown in Figure 1A. In some analyses, each subset was further divided into naive and activated/memory subsets by the expression of CD45RA. Expression of CD31, CD39, CD69, CD95, HLA-DR, FoxP3, Ki-67, and BCL-2 were determined on each T cell subset. Cell debris and doublets were excluded on the basis of side versus forward scatter. All cells were analyzed on a FACSCanto II (BD Biosciences) using FACSDiva (BD Biosciences) and FlowJo software (Tree Star).

For prospective monitoring of cohort 2, whole blood with EDTA anticoagulant was directly processed within 18 hours of sample collection using the Prep Plus 2 system (Beckman Coulter); rbc were lysed and leukocytes were fixed using TQPrep (Beckman Coulter) prior to analysis. Cells

were analyzed using a Cytomics FC 500 instrument and CXP Analysis 2.0 software (Beckman Coulter). Lymphocyte subsets were defined using the following antibodies: anti-CD3-PC5 (clone UCHT1; Beckman Coulter), anti-CD4-PC7 (clone SFC112T4D11; Beckman Coulter), and anti-CD25-FITC (clone B1.49.9; Beckman Coulter). In this study, Tregs and Tcons were defined as CD3⁺CD4⁺CD25^{med-hi} and CD3⁺CD4⁺CD25^{neg-lo}, respectively.

Cell sorting

For in vitro functional assays, specific cell populations were isolated by cell sorting using FACSARIA (BD Biosciences). Fresh PBMCs isolated after density gradient centrifugation from 30 ml whole blood were sorted into 4 different CD4⁺ T cell subsets defined by antigen expression patterns as follows: naive Tcons as CD4⁺CD45RA⁺CD25^{neg}CD127^{med-hi}, memory Tcons as CD4⁺CD45RA⁺CD25^{neg-dim}CD127^{med-hi}, naive Tregs as CD4⁺CD45RA⁺CD25^{med-hi}CD127^{lo}, and memory Tregs as CD4⁺CD45RA⁺CD25^{hi}CD127^{lo} (Figure 5A). Sorted cell populations were confirmed to be more than 95% pure.

In vitro proliferation assay

In vitro proliferation was measured using CFSE dilution. Freshly sorted CD4⁺ T cell subsets were labeled with CFSE (Invitrogen) according to manufacturer's directions. Briefly, cells were incubated at 37°C for 10 minutes with 5 μ M CFSE. Staining was stopped by adding RPMI 1640 containing 10% FBS at 4°C, followed by 1 wash in PBS. After labeling, cells were cultured in the presence of 0.1 μ g/ml anti-CD3 antibody (clone OKT3; eBioscience), 1 μ g/ml anti-CD28 antibody (clone L293; BD Biosciences), and 100 U IL-2 (BD Biosciences) in 96-well round-bottom plates at a concentration of 1×10^4 T cells per well. After 4 days, cells were harvested and incubated with anti-CD4 PC7 (clone SFC112T4D11; Beckman Coulter) and then analyzed for intracellular Foxp3-APC (clone PCH101; eBioscience). Cell division analysis of CD4⁺ cells was performed on FACScanto II (BD Biosciences). "Alive" cell gate and "apoptotic" cell gate were defined on the basis of side versus forward scatter. It was confirmed that over 85% of cells in "alive" cell gate were annexin V negative and over 90% of cells in "apoptotic" cell gate were annexin V positive.

Fas-induced apoptosis assay

CD4⁺ T cell subsets were cultured separately with 5 μ g/ml purified mouse anti-human CD95 antibody (clone EOS9.1; BD Biosciences) or with anti-CD95-free control medium in 96-well round-bottom plates at a concentration of 1×10^4 T cells per well. Apoptosis induction was measured 3, 6, and 18 hours after addition of anti-CD95. Cell death was assessed by annexin V/7-



AAD costaining and forward to side scatter profiles. Fas-specific cell death was calculated as follows: (percentage experimental cell death – percentage spontaneous cell death)/(100% – percentage spontaneous cell death) × 100.

Suppression assay

For suppression assay, freshly purified naive Tregs and memory Tregs were combined according to Treg subset balance of the patient. Tregs were cultured in 96-well round-bottom plates with 1×10^4 naive Tcons as responder cells from same donors and 5×10^4 irradiated (20 Gy) allogeneic PBMCs. After 5 days, cells were pulsed with ^3H thymidine for 16 hours. Incorporation of radioactivity was measured using a scintillation counter. Percentage suppression of proliferation was calculated as follows: percentage suppression = [(cpm responder cells alone – cpm responder/Tregs)/cpm responder alone] × 100.

Cytokine measurement

IL-2, IL-7, and IL-15 were measured in patient plasma by ELISA according to the manufacturer's instructions (Pierce Biotechnology; R&D Systems). Samples were obtained from cohort 2 patients at 3, 6, 9, 12, 18, and 24 months after HSCT and cryopreserved in aliquots before being analyzed. The number of samples analyzed at each time point was 15, 17, 24, 24, 12, and 15, respectively. We also studied plasma samples from 13 healthy adults.

Statistics

For 2-sample comparison of continuous variables, Wilcoxon's rank-sum test was performed. Fisher's exact test was used to compare categorical

variables. Wilcoxon's signed-rank test was used for difference of paired samples, and Spearman's rank test was used for correlation analysis. Various smoothing techniques were employed in graphical presentation of correlation. log-rank test was used for the comparison of the cumulative incidence of extensive chronic GVHD in Figure 7D. Multivariable linear models were constructed to identify clinical factors that affect levels of Treg and Tcon proliferation (Table 2). All tests performed were 2-sided and considered significant at the 0.05 level.

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