Low telomerase activity in CD4$^+$ regulatory T cells in patients with severe chronic GVHD after hematopoietic stem cell transplantation

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CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg) play an important role in the control of chronic graft-versus-host disease (cGVHD). In this study, we examined telomere length and telomerase activity of Treg and conventional CD4$^+$ T cells (Tcon) in 61 patients who survived more than 2 years after allogeneic hematopoietic stem cell transplantation. Cell proliferation and expression of Bcl-2 were also measured in each subset. Treg telomere length was shorter and Treg telomerase activity was increased compared with Tcon ($P < .0001$). After transplantation, Treg were also more highly proliferative than Tcon ($P < .0001$). Treg number, telomerase activity, and expression of Bcl-2 were each inversely associated with severity of cGVHD. These data indicate that activation of telomerase is not sufficient to prevent telomere shortening in highly proliferative Treg. However, telomerase activation is associated with increased Bcl-2 expression and higher Treg numbers in patients with no or mild cGVHD. In contrast, patients with moderate or severe cGVHD have fewer Treg with lower levels of telomerase activity and Bcl-2 expression. These results suggest that failure to activate Treg telomerase may restrict proliferative capacity and increase apoptotic susceptibility, resulting in the loss of peripheral tolerance and the development of cGVHD. (*Blood*. 2011;118(18): 5021-5030)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an effective and potentially curative treatment modality for patients with various hematologic malignancies and nonmalignant diseases. With advances in supportive care, infection prophylaxis, and development of less toxic conditioning regimens, overall outcomes have improved significantly in the past 2 decades.[1] Nevertheless, chronic graft-versus-host disease (cGVHD) continues to be one of the major complications of allogeneic HSCT, occurring in 40% to 70% of patients.[2,3] cGVHD thus causes significant mortality and morbidity that impairs the quality of life in long-term survivors.

Regulatory T cells (Treg) expressing CD4, CD25, and Foxp3 play an important role in the maintenance of self tolerance and immune homeostasis in healthy persons.[4] CD4 Treg abnormalities are known to contribute to the development of autoimmune disorders, allergy, cancer, and aplastic anemia.[5-8] Treg develop in the thymus, and studies in model systems have demonstrated that poor reconstitution of Treg after allogeneic HSCT results in an increased incidence of GVHD.[9-11] In humans, we and others previously reported that patients with cGVHD have reduced frequency of circulating Treg compared with patients without active cGVHD and healthy persons.[12,13] We also demonstrated that abnormalities of Treg reconstitution and homeostasis in the first year after transplantation contribute to the subsequent development of cGVHD. During this period of immune reconstitution, Treg undergo high levels of proliferation, but expansion of the Treg population in vivo is limited by increased susceptibility to apoptosis.[14] The inability to maintain peripheral expansion of Treg is associated with a high incidence of severe cGVHD, but the cellular mechanisms that promote susceptibility to apoptosis in proliferating Treg have not been identified.

Telomeres, located at the ends of all chromosomes, are complex structures consisting of tandem hexanucleotide repeats of the DNA sequence TTAGGG and associated proteins, termed the shelterin complex. Telomere DNA gradually shortens with each cell division as a result of failure to completely replicate the 3′ end of chromosomes by DNA polymerase.[15,16] With critical telomere shortening, cells become senescent or undergo apoptosis. Short telomeres also cause genomic instability, which can lead to end-to-end chromosome fusions, translocations, and malignant transformation.[17-19] In actively dividing cells, telomere length is maintained by telomerase, a ribonucleoprotein complex enzyme that adds telomere DNA to the end of chromosomes.[20] The telomerase complex consists of telomerase reverse transcriptase, its integral RNA template, the protein dyskerin, and other associated proteins that stabilize the complex.[21] Telomerase is active in germline cells, stem cells, and somatic cells with high replicative demands, such as hematopoietic cells and lymphocytes.[22,23] Previous studies have reported shortening of telomere length in peripheral blood mononuclear cells, including T lymphocytes in patients after allogeneic HSCT. Telomere shortening in this setting has been attributed to excessive replication cycles of donor-derived hematopoietic stem cells, suggesting that endogenous telomerase activity in hematopoietic stem cells is not sufficient to prevent proliferation-associated loss of telomere DNA.[24-26]

Previous studies of CD4 Treg in healthy persons have demonstrated that these cells are highly proliferative in vivo and have

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increased telomerase activity. Nevertheless, proliferating CD4 Treg have relatively short telomeres and also exhibit increased susceptibility to apoptosis.[27] Patients with cancer often have increased numbers of circulating Treg. In this setting, proliferating Treg have shortened telomeres despite the presence of increased levels of telomerase.[28] To examine the potential role of telomerase induction in the maintenance of CD4 Treg after allogeneic HSCT, we examined telomere length and telomerase activity in purified T-cell subsets from 61 patients >2 years after transplantation. Patient Treg were highly proliferative, and relative telomere length was significantly shorter in Treg from transplantation patients compared with healthy subjects. Importantly telomerase activity in Treg was inversely associated with severity of cGVHD and Treg number. Telomerase activation in Treg is also associated with increased expression of the antiapoptotic protein Bcl-2. Thus, in patients with no or mild cGVHD, activation of telomerase in Treg was associated with increased expression of Bcl-2, higher numbers of Treg, and establishment of peripheral tolerance. In contrast, failure of telomerase activation in Treg in patients with severe cGVHD appears to increase apoptotic susceptibility of these cells, resulting in fewer Treg and the loss of peripheral tolerance.

Methods

Patients and samples

All patients included in this cross-sectional study were enrolled in clinical protocols approved by the Institutional Review Board of the Dana-Farber/Harvard Cancer Center. Written informed consent was obtained from each patient before sample collection in accordance with the Declaration of Helsinki. Blood samples were obtained from 61 patients who underwent allogeneic HSCT at the Dana-Farber Cancer Institute and Brigham and Women's Hospital (Boston, MA) and survived without relapse for >2 years after HSCT. The diagnosis of cGVHD was based on characteristic manifestations and pathologic findings. The severity of cGVHD was classified at the time of sampling according to the National Institute of Health Consensus recommendations.[29] In patients receiving immunosuppressive therapy, drug doses were not stopped or modified in the 3-month period before samples were obtained for this study. For comparison, samples from 19 healthy subjects who are age matched to the patient's donor age at the time of analysis were also examined.

PBMCs were isolated from blood samples by density gradient centrifugation (Ficoll-Paque; GE Healthcare) and cryopreserved in aliquots until needed for cell sorting, flow cytometric analysis, and functional assays.

Cell sorting

Frozen PBMCs were thawed, washed 2 times with PBS, and incubated with the following 3 antibodies for 20 minutes at room temperature: anti-CD4 Pacific blue (clone RPA-T4; BD Biosciences), anti-CD25 PE-Cy7 (clone M-A251; BD Biosciences), and anti-CD127 PE (clone HIL-7R-M21; BD Bioscience). After incubation, cells were washed and isolated by cell sorting using BD FACSAria II Cell Sorter (BD Biosciences) into 2 different $CD4^+$ T-cell subsets defined by antigen expression patterns as follows: $CD4^+$ conventional T cells (Tcon) as $CD4^+$ $CD25^{neg\text{-}low}CD127^{med\text{-}hi}$, Treg as $CD4^+CD25^{med\text{-}hi}CD127^{low}$, respectively. Sorted cell populations were confirmed to be $>95\%$ pure.

Treg suppression assay

A total of 20 000 purified Tcon were cocultured with the same number of autologous Treg in DMEM supplemented with 10% FCS, 2mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For polyclonal stimulation, anti-CD3 (clone OKT-3; eBioscience) was immobilized on round-bottom 96-well plates at 1 μg/mL in the presence of 10^5 irradiated allogeneic PBMCs as antigen-presenting cells. Cultures of Tcon alone with and without immobilized anti-CD3 and PBMCs were used as controls for the following assays. After 5 days, ^{3}H-thymidine (PerkinElmer Life and Analytical Sciences) was added at 1 μCi/well and cultured for an additional 20 hours. Incorporation of radioactivity was calculated in triplicate using a liquid scintillation counter. To examine specific suppression of Tcon proliferation, purified Tcon were labeled with 5μM CFSE (Invitrogen) for 10 minutes at 37°C. A total of 20 000 CFSE-labeled Tcon were cocultured with an equal number of unlabeled autologous Treg as described earlier in this paragraph. After 5 days, cells were harvested and stained with anti-CD4 Pacific blue, anti-CD25 PE-Cy7, and anti-CD127 APC eFluor780 (clone eBioRDR5; eBioscience). CFSE dilution of Tcon was analyzed on a BD FACS Canto II (BD Biosciences) using FACSDiva (BD Biosciences) and FlowJo 7.6.1 software (TreeStar).

DNA extraction

Genomic DNA of sorted Tcon and Treg was extracted using QIAGEN FlexiGene DNA Kit (QIAGEN) according to the manufacturer's protocol. After extraction, the Quant-iT dsDNA HS assay kit and Qubit fluorometer (Invitrogen) were used to quantify genomic DNA. Genomic DNA was stored in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 7.5) at 4°C at a concentration of <20 ng/μL. DNA stocks were diluted into pure water before starting real-time PCR runs described in "Measurement of telomere length."

Measurement of telomere length

Relative average telomere length was examined by the real-time PCR-based telomere assay described previously.[30] Briefly, 15 μL master mix containing either the telomere or β-globin PCR reaction mixture was mixed into 15 μL of each experimental DNA sample, containing the same amount of genomic DNA diluted in pure water. The telomere reaction mixture consisted of 1× QIAGEN Quantitect Sybr GreenPCR Master Mix, 2.5mM of DTT (USB Corporation), 100nM of Tel-1b primer (CGGTTT-GTTTGGGTTTGGGTTTGGGTTTGGGTT), and 900nM of Tel-2b primer (GGCTTGCCTTACCCTTACCCTTACCCTTACCCTAC-CCT). Telomere PCR was performed at 95°C for 5 minutes, followed by amplification rounds consisting 40 cycles at 95°C for 15 seconds and 54°C for 1 minute with signal acquisition. The β-globin reaction mixture consisted of 1× QIAGEN Quantitect SYBR GreenPCR Master Mix, 300nM of hbg1 primer (GCTTCTGACACAACTGTGTTCACTAGC), and 700nM of hbg2 primer (CACCAACTTCATCCACGTTCACC). The β-globin PCR was performed at 95°C for 5 minutes, followed by amplification rounds consisting 40 cycles at 95°C for 15 seconds, 58°C for 20 seconds, and 72°C for 28 seconds with signal acquisition. The telomere repeat copy number to single gene copy number (T/S) ratio was determined using an Applied Biosystems 7300 thermocycler in a 96-well format. All samples for both the telomere and single-copy gene β-globin reactions were done in triplicate. In addition to the samples, each 96-well plate contained a 6-point standard curve from 1 to 100 ng using genomic DNA derived from Jurkat cells. The T/S ratio for a DNA sample is T, the number of nanograms of the standard Jurkat cells DNA that matches the sample for copy number of the telomere template, divided by S, the number of nanograms of the standard DNA that matches the sample for copy number of the β-globin. Three T/S results were obtained for each sample, and the final reported result for a sample in a given run is the average of the 3 T/S values. Average T/S is expected to be proportional to the average telomere length per cell.

Measurement of telomerase activity

Telomerase activity was examined using the Telo TAGGG Telomerase PCR ELISA PLUS kit (Roche Diagnostics) according to the manufacturer's instructions. Sorted Tcon and Treg were lysed using lysis buffer at a concentration of 500 cells per microliter. After incubation for 30 minutes on ice, cell lysates were centrifuged and 3 μL of cell extract (corresponding to 1.5×10^3 cell equivalents) was used for each telomeric repeat PCR amplification reaction. The PCR amplification product was then denatured and hybridized to digoxigenin-labeled detection probes. The resulting products were immobilized via the biotin label to streptavidin-coated

Table 1. Patient characteristics

	No cGVHD (n = 13)	Mild cGVHD (n = 20)	Moderate cGVHD (n = 16)	Severe cGVHD (n = 12)	*P*
Age, y					NS
Median	55	62	55	61	
Range	36-71	32-73	26-72	38-71	
Sex					NS
Male	9 (69)	13 (65)	9 (57)	6 (50)	
Female	4 (31)	7 (35)	7 (43)	6 (50)	
Diseases					NS
AML	4 (31)	9 (45)	5 (32)	4 (34)	
ALL	1 (7)	2 (10)	2 (12)	0 (8)	
CML	1 (7)	1 (5)	1 (6)	1 (8)	
CLL	4 (31)	2 (10)	1 (6)	3 (25)	
NHL	3 (24)	4 (20)	6 (38)	0 (0)	
MDS	0 (0)	1 (5)	1 (6)	3 (25)	
MF	0 (0)	1 (5)	0 (0)	0 (0)	
AA	0 (0)	0 (0)	0 (0)	1 (8)	
Conditioning regimen				NS	
Myeloablative	2 (15)	5 (25)	6 (37)	3 (25)	
Nonmyeloablative	11 (85)	15 (75)	10 (63)	9 (75)	
Donor source				NS	
MRD	4 (31)	7 (35)	7 (43)	3 (25)	
MUD	9 (69)	13 (65)	9 (57)	9 (75)	
Stem cell source				NS	
BMT	1 (7)	2 (10)	1 (6)	0 (0)	
PBSCT	12 (93)	18 (90)	15 (94)	12 (100)	
Donor age at sampling, y				NS	
Median	42	43	39	40	
Range	24-57	26-67	23-60	23-57	
Sex matching (recipient/donor)			NS		
Male/male	6 (47)	9 (45)	5 (31)	3 (25)	
Male/female	3 (23)	4 (20)	4 (25)	3 (25)	
Female/male	2 (15)	4 (20)	5 (31)	3 (25)	
Female/female	2 (15)	3 (15)	2 (13)	3 (25)	
Medication					
Predonisone	1 (8)	13 (65)	11 (69)	11 (92)	NS
Tacrolimus	2 (16)	5 (25)	4 (25)	7 (59)	NS
Sirolimus	1 (8)	4 (20)	2 (13)	7 (59)	.02
MMF	0 (0)	1 (5)	4 (25)	4 (33)	NS
ECP	0 (0)	3 (15)	1 (7)	3 (25)	NS
Acute GVHD prophylaxis					
Tacrolimus/sirolimus	3 (24)	6 (30)	4 (25)	3 (25)	
Tacrolimus/MTX	1 (8)	5 (25)	5 (31)	1 (8)	
Tacrolimus/sirolimus/MTX	(62)	9 (45)	6 (38)	6 (50)	
Others	1 (8)	0 (0)	1 (6)	2 (17)	
Acute GVHD grade 2-4					NS
No	7 (54)	16 (80)	9 (56)	7 (58)	
Yes	6 (46)	4 (20)	7 (44)	5 (42)	
cGVHD target organ					
Skin	0 (0)	15 (75)	10 (63)	10 (84)	NS
Mouth	0 (0)	5 (25)	9 (57)	6 (50)	NS
Eye	0 (0)	3 (15)	6 (37)	9 (75)	.003
Liver	0 (0)	0 (0)	4 (25)	3 (25)	.054
Lung	0 (0)	0 (0)	2 (13)	2 (17)	NS
Muscle	0 (0)	4 (20)	3 (19)	5 (42)	NS
Joint and fascia	0 (0)	1 (5)	0 (0)	2 (17)	NS
Median time after transplantation, days					
Median	1249	1503	1207	1208	NS
Range	856-2711	757-2739	754-2768	772-3492	

All *P* values indicate comparisons among 4 cGVHD groups, except for medication and cGVHD target organ, among mild, moderate, and severe cGVHD groups. Values in parentheses are percentages.

NS indicates not significant; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndrome; MF, myclofibrosis; AA, aplastic anemia; MRD, matched related donor; MUD, matched unrelated donor; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; MMF, mycophenolate mofetil; ECP, extracorporeal photopheresis; and MTX, methotrexate.

Table 2. Median number of CD4$^+$ T cell, Tcon, and Treg in peripheral blood

	n	CD4$^+$ T cell	*P*	Tcon	*P*	Treg	*P*	Treg/Tcon ratio	*P*
Healthy subjects	19	833	.0002*	780	.0003*	39	.0001*	0.04	NS*
Patients	61	504	.0042†	469	.0043†	19	.0023†	0.04	NS†
No cGVHD	13	583	—	566	—	28	—	0.05	—
Mild cGVHD	20	548	—	522	—	20	—	0.03	—
Moderate cGVHD	16	386	—	381	—	17	—	0.04	—
Severe cGVHD	12	285	—	261	—	9	—	0.04	—

NS indicates not significant; and —, not applicable.

**P* values indicate comparison for healthy subjects versus all patients.

†*P* values indicate comparison among cGVHD groups.

96-well microplate. Immobilized amplicons were then detected with an antibody against digoxigenin that is conjugated to HRP and the sensitive peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine. After incubation for 20 minutes at room temperature, stop reagent was added and sample values were measured as absorbance at 450 nm read against the blank value (reference wavelength 690 nm). All samples were tested in duplicate, and the result for a sample is the average of the sample values. PCR products from 15 samples were also analyzed for telomerase activity by a Southern hybridization technique previously reported.[31] In brief, 20 μL of PCR products was mixed with 5 μL of 5× sample buffer (Invitrogen) dye and resolved on PAGE using 10% Novex TBE Gels (Invitrogen), and transferred to nylon membranes. After incubating the membrane with a streptavidin alkaline phosphatase conjugate (Roche Diagnostics), chemiluminescence techniques were used to visualize the blotted products.

Flow cytometric analysis of Ki-67, Bcl-2, and Foxp3

To detect intracellular Ki-67, Bcl-2, and Foxp3, PBMCs were first incubated with anti-CD4 Pacific blue, anti-CD25 PE-Cy7, and anti-CD127 APC eFluor780. For Ki-67 and Bcl-2 staining, cells were then washed twice in PBS and suspended in 100 μL of Fixation/Permeabilization solution (BD Biosciences) and incubated for 20 minutes at 4°C. After fixation, cells were washed and incubated with anti-Ki-67 PE (clone B56; BD Biosciences), or anti-Bcl-2 PE (clone Bcl-2/100; BD Biosciences) or isotype-matched IgG-PE (clone MOPC-21; BD Biosciences) for 30 minutes at 4°C. For Foxp3 staining, surface-stained PBMCs were suspended in fixation and permeabilization buffer (eBioscience) and then incubated with anti-Foxp3 APC (clone PCH101; eBioscience) or isotype-matched IgG-APC (eBioscience) for 30 minutes at 4°C. After incubation and washing, stained cells were analyzed on the FACSCanto II using FlowJo 7.6.1 software. Cell debris and doublets were excluded on the basis of side versus forward scatter. Ki-67 positivity and Foxp3 expression were determined for each Tcon and Treg subset. Relative Bcl-2 expression in each Tcon and Treg was calculated by dividing the median fluorescence intensity (MFI) for Bcl-2–PE by the median value of MFI for the isotype matched IgG.

Statistical methods

Descriptive statistics were used for patient and transplant-related characteristics. Fisher exact test or a χ^2 test was used for group comparisons for categorical variables. The Wilcoxon-rank-sum test or the Kruskal-Wallis test was performed for 2 or more group comparisons for continuous variables. The Wilcoxon signed-rank test was used for difference of paired samples for continuous variables. Spearman rank test was used for correlation analysis, and super-smoother, a smoothing technique, in R (Version 2.10.1) was used to describe the correlation between telomerase activity and Treg and Tcon cell number. Stratified Wilcoxon rank-sum test was used to assess the influence of immunosuppressive agents on telomerase activity. Multivariable Cox regression analysis was performed to investigate the effect of telomere length and telomerase activity on cGVHD after adjusting for patients and transplant characteristics, such as age, patient sex, disease, conditioning regimen, donor age at sampling, stem cell source, immunosuppressive agent, grade 2 to 4 acute GVHD, and donor source. All tests were 2-sided at the significance level of .05, and multiple comparisons were not adjusted.

Results

Patients

Clinical characteristics of the 61 patients included in this study are summarized in Table 1. At the time of sample analysis, 13 patients had no cGVHD, 20 had mild cGVHD, 16 had moderate cGVHD, and 12 had severe cGVHD. Eight patients with no cGVHD had a previous history of cGVHD that had resolved. Patient samples were obtained at a median of 43 months after HSCT, and 43 of 61 patients (70%) were receiving immunosuppressive agents at the time of sample collection. Thirty-six of 61 patients (59%) were receiving steroids. The median patient age was 58 years (range, 26-73 years), and the median donor age at the time of sampling was 41 years (range, 23-67 years). Thirty-seven patients were male, and 14 of them received stem cells from female donors. Fifty-seven patients received filgrastim-mobilized peripheral blood stem cell grafts and 4 received bone marrow stem cells. Blood samples were also obtained from 19 adult healthy subjects (median, 43 years; range, 24-61 years).

Number of CD4 Tcon and CD4 Treg in patient samples

The total numbers of CD4 T cells, Tcon, and Treg in peripheral blood were enumerated by flow cytometry in each patient sample and compared with healthy subjects (Table 2). The number of CD4$^+$ T cells, Tcon, and Treg in patients after allogeneic HSCT was decreased compared with healthy subjects ($P = .0002$, $P = .0003$, and $P < .0001$, respectively), and these numbers were inversely associated with severity of cGVHD ($P = .0042$, $P = .0043$, and $P = .0023$, respectively). However, the ratio of Treg to Tcon in patients was not significantly different from healthy subjects and between each cGVHD group.

Treg phenotype and immune regulatory function after allogeneic HSCT

To further characterize the phenotype and suppressive function of CD4 Treg and Tcon in patient samples, CD4$^+$CD25$^{\text{med-hi}}$CD127$^{\text{low}}$ and CD4$^+$CD25$^{\text{low}}$CD127$^{\text{hi}}$ populations were purified by cell sorting using gates shown in Figure 1A. As shown in a representative example (Figure 1B), intracellular Foxp3 expression was examined in each population by flow cytometry and was found to be expressed at high levels only in the CD4$^+$CD25$^{\text{med-hi}}$CD127$^{\text{low}}$ Treg subset. In vitro suppression assays were also performed with purified Tcon and Treg using both ^{3}H-thymidine incorporation and CFSE dye dilution as measures of T-cell proliferation. In both assays, Tcon proliferation was inhibited by coculturing with autologous Treg (Figure 1C-D), indicating that purified

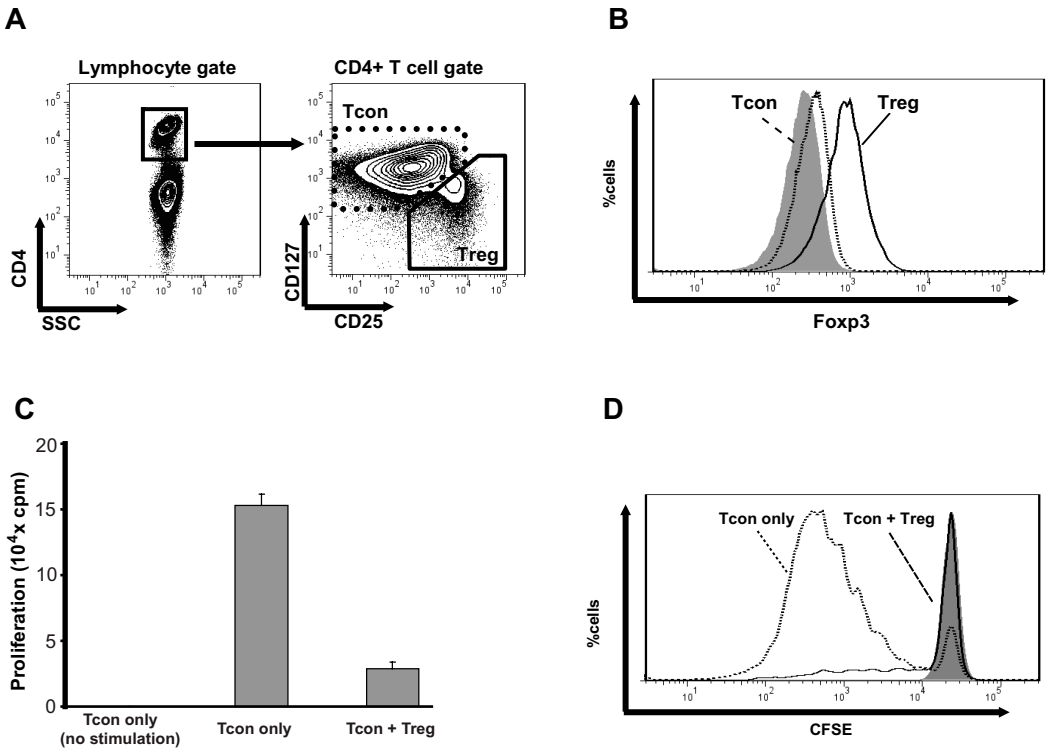

Figure 1. Phenotype of Treg and Tcon, and suppressive activity of Treg. (A) Representative contour plot showing expression of CD4 in the lymphocyte gate, and expression of CD25 and CD127 in the $CD4^+$ lymphocyte gate. PBMCs were stained with CD4, CD25, and CD127 antibody. $CD4^+$ lymphocytes were isolated into $CD25^{neg\text{-}low}CD127^{med\text{-}high}$ Tcon (dotted line), and $CD25^{med\text{-}high}CD127^{low}$ Treg (solid line). (B) Expression of Foxp3 in Treg and Tcon. Surface-stained PBMCs were stained with intracellular anti-Foxp3 antibody or isotype IgG (filled histograms). Foxp3 expression was measured in Tcon (dotted line) and Treg (solid line). (C-D) Functional capacity of isolated Treg. Tcon and Treg were purified by flow cytometric cell sorting. A total of 2×10^4 Tcon alone or cocultured with 2×10^4 autologous Treg were incubated with 10^5 irradiated allogeneic PBMCs in the presence of anti-CD3 antibody. Tcon cultured alone in the absence of allogeneic PBMCs and anti-CD3 antibody was used as a negative control. Tcon cultured with allogeneic PBMC and anti-CD3 antibody was used as the positive control. Proliferation of Tcon was determined by ^{3}H-thymidine incorporation (C) and CFSE dilution assay (D). Representative FACS profile of Tcon only (dotted line), Tcon cocultured with Treg (solid line), and Tcon only without stimulation (filled histograms) is shown. Both assays were performed in triplicate.

$CD4^+CD25^{med\text{-}hi}CD127^{low}$ Treg maintain expected levels of suppressive function.

Short telomere length of Treg in patients after HSCT

Relative telomere length was measured by real-time PCR in Treg and Tcon subsets purified by cell sorting. As shown in Figure 2A, telomere length of Treg was significantly shorter compared with Tcon in both healthy subjects and patients after HSCT (0.26 ± 0.08 vs 0.32 ± 0.06, $P < .0001$; and 0.11 ± 0.07 vs 0.24 ± 0.09, $P < .0001$, respectively). Moreover, telomere length in both Tcon and Treg subsets in patients after HSCT was shorter compared with healthy subjects ($P = .001$ and $P < .0001$, respectively; Figure

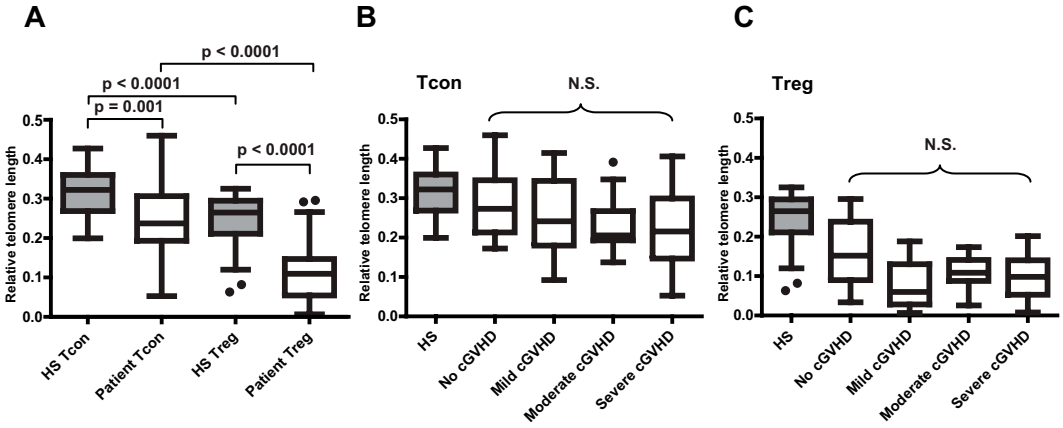

Figure 2. Measurement of telomere length in Tcon and Treg. Tcon and Treg were purified by cell sorting, and DNA from each population was isolated and used as a template for quantitative real-time PCR for telomere length measurement. Relative telomere length indicates the average T/S of each sample described in "Measurement of telomerase length." All samples were measured in triplicate. Box plots indicate the values for median, 25th and 75th percentile; whiskers, outer part of distribution; and outside dots, individual outliers. At the time of sample analysis, 13 patients had no cGVHD, 20 had mild cGVHD, 16 had moderate cGVHD, and 12 had severe cGVHD. Samples were also obtained from 19 adult healthy subjects (HS). (A) Telomere length of Tcon and Treg in healthy subjects and patients after HSCT. *P* values indicate comparisons for each category. (B) Telomere length of Tcon in healthy subjects and patient subsets based on severity of cGVHD. *P* values indicate comparisons among patient subsets. (C) Telomere length of Treg in healthy subjects and patient subsets based on severity of cGVHD. *P* values indicate comparisons among patient subsets. N.S. indicates not significant.

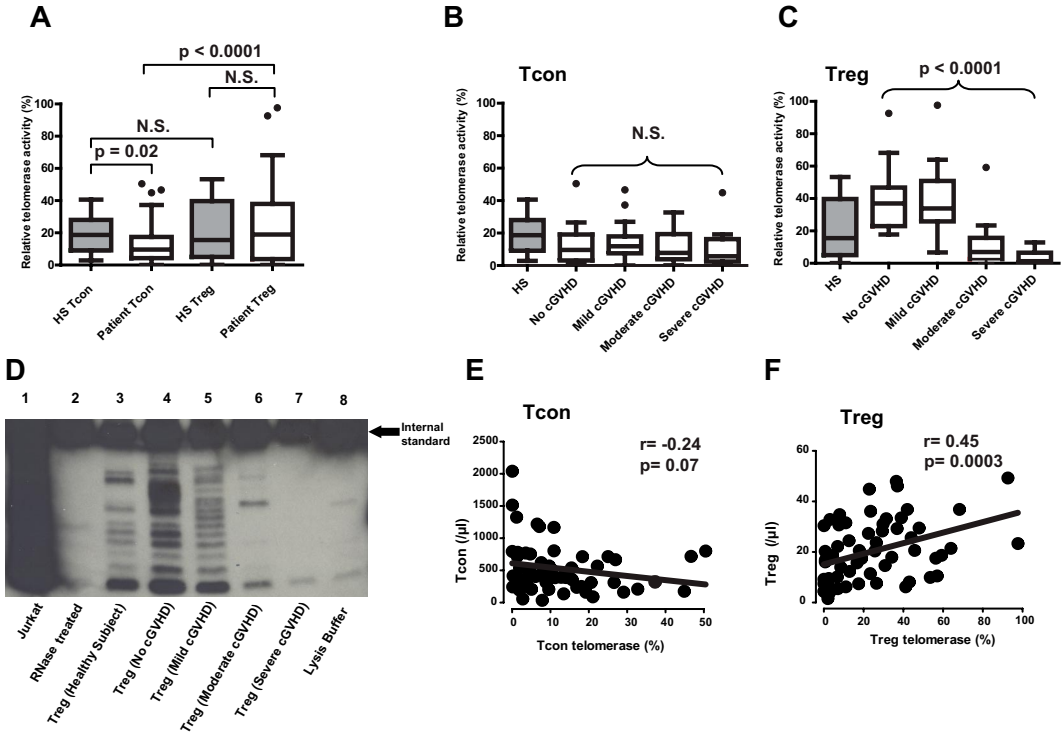

Figure 3. Measurement of telomerase activity in Tcon and Treg. Telomerase activity was measured in Tcon and Treg by PCR ELISA and southern hybridization. The level of telomerase activity is shown in percentage as relative telomerase activity compared with that of control template measured in each kit. All samples were measured in duplicate. (A) Telomerase activity of Tcon and Treg in healthy subjects (HS) and patients after HSCT. *P* values indicate comparisons for each category. (B) Telomerase activity of Tcon in healthy subjects and patient subsets based on severity of cGVHD. N.S. indicates not significant. (C) Telomerase activity of Treg in healthy subjects and patient subsets based on severity of cGVHD. (B-C) *P* values indicate comparisons among patient subsets. (D) Representative southern hybridization experiment for telomerase activity. Internal standard indicates the control for PCR amplification of telomere extension product whose size is 216 bp. The value for relative telomerase activity (%) calculated by PCR-ELISA is 1150.78 (lane 1, Jurkat cells), 23.76 (lane 3, Treg in healthy subject), 47.91 (lane 4, Treg in patient with no cGVHD), 31.37 (lane 5, Treg in patient with mild cGVHD), 7.58 (lane 6, Treg in patient with moderate cGVHD), and 0.09 (lane 7, Treg in patient with severe cGVHD), respectively. (E-F) Relationship between telomerase activity and each cell number in Tcon (E) and Treg (F). Correlation analysis was calculated using Spearman rank test.

2A), suggesting that both Tcon and Treg subsets have undergone extensive cell division after HSCT. However, within both Tcon and Treg subsets, there was no correlation between telomere length and severity of cGVHD (Figure 2B-C).

Treg telomerase activity is inversely correlated with severity of cGVHD

Telomerase activity was examined by PCR-ELISA and Southern blotting. Telomerase activity of Treg in patients after HSCT was increased compared with Tcon (9.66 ± 11.75 vs 18.96 ± 23.09, $P < .0001$; Figure 3A), although there was no significant difference in telomerase activity between Tcon and Treg in healthy subjects (18.73 ± 11.13 vs 15.58 ± 18.62, $P = .68$; Figure 3A). Telomerase activity of Tcon in patients after HSCT was decreased compared with healthy subjects ($P = .02$), but decreased telomerase activity in Tcon was not correlated with severity of cGVHD (Figure 3B). In contrast, telomerase activity of Treg in patients with no or mild cGVHD was higher compared with healthy subjects (no cGVHD, $P = .014$; mild cGVHD, $P = .0056$, respectively). Moreover, telomerase activity of Treg in patients with moderate cGVHD was slightly lower compared with healthy subjects ($P = .066$), and telomerase activity of Treg in patients with severe cGVHD was significantly lower compared with healthy subjects ($P = .0008$). As a result, telomerase activity in patient Treg was significantly associated with severity of cGVHD ($P < .0001$; Figure 3C). To confirm the results of telomerase activity measured by PCR-ELISA, telomerase activity in some Treg PCR samples was also examined by Southern blotting (Figure 3D). We next examined the correlation between telomerase activity and the absolute number of Tcon and Treg in each patient sample. As shown in Figure 3E, there was no correlation between Tcon telomerase activity and Tcon cell number ($r = -0.24$, $P = .07$), but telomerase activity in patient Treg was associated with Treg cell number ($r = 0.45$, $P = .0003$; Figure 3F).

Increased Treg proliferation in patients with cGVHD

To define the relationship between telomerase activation and cell proliferation, we examined the expression of Ki-67 within Tcon and Treg subsets in patients and healthy subjects (Figure 4). Cell proliferation, measured by Ki-67 expression, was significantly increased in both Tcon and Treg subsets in patients with cGVHD compared with healthy subjects (Tcon, $0.6\% \pm 0.4\%$ vs $0.2 \pm 0.1\%$, $P < .0001$; Treg, $5.2\% \pm 1.4\%$ vs $2.9\% \pm 0.9\%$, $P < .0001$, respectively; Figure 4A). In addition, Ki-67 expression was higher in Treg compared with Tcon in both healthy subjects and patients ($P = .0002$ and $P < .0001$, respectively; Figure 4A). These results indicate that Treg undergo higher levels of proliferation than Tcon and this difference is amplified after HSCT. However, Ki-67 expression in Tcon and Treg subsets was not associated with severity of cGVHD (Figure 4B-C) and also not associated with the level of telomerase activation in either subset (Tcon, $r = 0.05$, $P = .69$; Treg, $r = 0.12$, $P = .37$, respectively; Figure 4D-E).

Bcl-2 expression of Treg in patients with cGVHD

Although the induction of telomerase activity in both Tcon and Treg after transplantation does not prevent overall telomere shortening in these cells, there may be other mechanisms whereby induction of

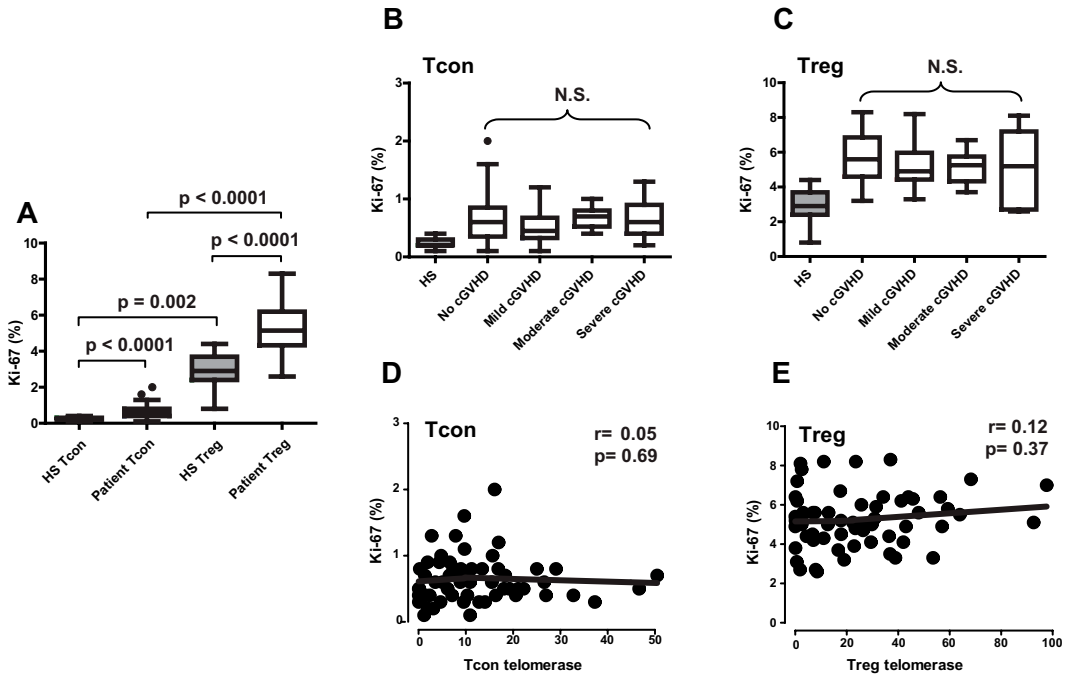

Figure 4. Cell proliferation of Tcon and Treg. The proliferation of Tcon and Treg subsets was determined by expression of Ki67 by flow cytometry. (A) Cell proliferation of Tcon and Treg in healthy subjects (HS) and patients after HSCT. *P* values indicate comparisons for each category. (B) Cell proliferation of Tcon in healthy subjects and patient subsets based on severity of cGVHD. (C) Cell proliferation of Treg in healthy subjects and patient subsets based on severity of cGVHD. (B-C) *P* values indicate comparisons among patient subsets. N.S. indicates not significant. (D-E) Relationship between telomerase activity and cell proliferation in Tcon (D) and Treg (E) populations. Correlation was calculated using Spearman rank test.

telomerase may enhance cell survival. To assess the potential effect of telomerase activity on susceptibility to apoptosis, intracellular expression of Bcl-2 was determined by flow cytometry in Treg and Tcon. Bcl-2 expression in Treg was lower compared with Tcon in patients after HSCT and healthy subjects (patients Treg vs Tcon, 33.7 ± 10.9 vs 39.4 ± 11.6: $P < .0001$; healthy subjects Treg vs Tcon, 32.5 ± 4.3 vs 36.53 ± 4.1: $P = .008$, respectively; Figure 5A). Moreover, expression of Bcl-2 in Tcon was similar in all patient groups (Figure 5B). In contrast, Bcl-2 expression in Treg from patients with no or mild cGVHD was higher compared with healthy subjects (no cGVHD, $P = .02$; and

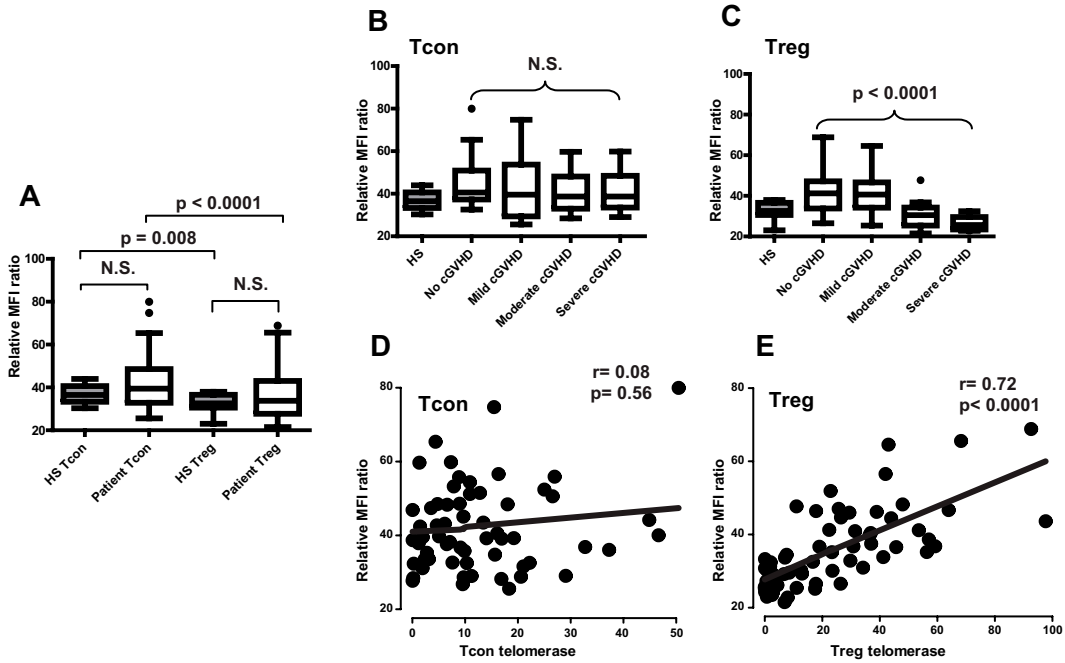

Figure 5. Correlation of Bcl-2 protein expression, severity of cGVHD, and telomerase activity. Bcl-2 expression of Tcon and Treg was measured by flow cytometry. Relative Bcl-2 expression was calculated by dividing the median MFI of Bcl-2 by the median MFI of isotype matched IgG. (A) Bcl-2 expression of Tcon and Treg in healthy subjects (HS) and patients after HSCT. *P* values indicate comparisons for each category. (B) Bcl-2 expression of Tcon in healthy subjects and patient subsets based on severity of cGVHD. N.S. indicates not significant. (C) Bcl-2 expression of Treg in healthy subjects and patient subsets based on severity of cGVHD. (B-C) *P* values indicate comparisons among patient subsets. (D-E) Relationship between telomerase activity and each Bcl-2 expression in Tcon (D) and Treg (E). Correlation was calculated using Spearman rank test.

mild cGVHD, $P = .006$, respectively), and Bcl-2 expression in Treg from patients with severe cGVHD was lower compared with healthy subjects ($P = .0026$). As shown in Figure 5C, Bcl-2 expression in patient Treg was inversely associated with severity of cGVHD ($P < .0001$). With this finding, we also examined the correlation between telomerase activity and Bcl-2 expression. Bcl-2 expression in Tcon was not associated with telomerase activity ($r = 0.08$, $P = .56$; Figure 5D). In contrast, there was a strong positive correlation between telomerase activity and Bcl-2 expression in Treg ($r = 0.72$, $P < .0001$; Figure 5E). Thus, in patients with no or mild cGVHD, Treg express both higher levels of Bcl-2 and telomerase activity. Both of these factors together probably contribute to increased resistance to apoptosis resulting in increased cell number.

Discussion

Successful allogeneic HSCT is characterized by the full reconstitution of normal donor lymphopoiesis as well as hematopoiesis and the establishment of immune tolerance to recipient tissues. $CD4^+$ regulatory T cells play an important role in the establishment and maintenance of peripheral tolerance, and previous studies in murine models and patients with GVHD have reported that inadequate reconstitution of these cells is associated with the development of acute GVHD and cGVHD.[9,12,32] To define the mechanisms responsible for inadequate reconstitution of Treg in some persons, previous studies in our laboratory examined several factors that contribute to Treg homeostasis in the first year after HSCT.[14] These studies demonstrated that thymic generation of naive Treg was markedly impaired during this period, and reconstitution of Treg was primarily dependent on high levels of proliferation and expansion of Treg with a "memory" phenotype in vivo. However, highly proliferative Treg were also more susceptible to apoptosis. After transplantation, Treg appeared to mediate normal suppressive functions, but in some patients, increased homeostatic proliferation of Treg was not sufficient to overcome shortened survival and adequate numbers of circulating Treg could not be maintained. These patients did not appear to be able to establish tolerance to recipient tissues and were at high risk for developing severe cGVHD.[12-14]

To determine whether inadequate activation of telomerase could contribute to the shortened survival of highly proliferative Treg, we examined telomere length and telomerase activity in a cross-sectional cohort of 61 patients who had survived without relapse for at least 2 years after allogeneic HSCT. Telomere length and telomerase activity were measured independently in purified CD4 Treg and CD4 Tcon. For comparison, similar studies were carried out in 19 age-matched healthy subjects. These studies demonstrated that telomere length in both Tcon and Treg was shorter in patients after HSCT compared with healthy subjects. This observation is consistent with previous studies that found relatively short telomeres in all donor-derived hematopoietic cells after transplantation, reflecting the marked expansion of hematopoietic stem cells required for reconstitution of donor hematopoiesis in these patients.[24-26] We also found that telomere length of Treg was shorter compared with Tcon in both healthy subjects and patients after HSCT. This finding suggests that Treg normally undergo more cell divisions than Tcon as these cells differentiate from T-cell precursors and expand in response to homeostatic signals. This difference is amplified after stem cell transplantation and is consistent with our previous finding that Treg reconstitution is highly dependent on peripheral expansion. Although both Tcon and Treg have shorter telomeres after HSCT compared with healthy subjects, telomere length in these T-cell subsets was not correlated with severity of cGVHD.

Telomerase is normally induced in highly proliferative cells, including activated T cells, to protect these cells from telomere shortening and premature senescence.[22,23,33] Early after HSCT, both Tcon and Treg undergo intense homeostatic proliferation to reconstitute normal numbers of CD4 T cells. Our analysis of patients after HSCT focused on persons with active cGVHD and was carried out with samples obtained ~ 3.5 years after transplantation. Results at this relatively late time after HSCT indicate that the previous level of telomerase activation was not sufficient to prevent overall telomere shortening in both Treg and Tcon. Moreover, at this time, telomerase activity in Tcon was significantly less in patients than in healthy subjects (Figure 3A), even though the level of proliferation remained significantly higher (Figure 4A). Telomerase activity was significantly higher in patient Treg compared with patient Tcon. However, Treg telomerase activity varied widely in patient samples. When examined in relationship to clinical status, telomerase activity was increased in Treg in patients with no or mild cGVHD and decreased in Treg in patients with moderate and severe cGVHD compared with healthy subjects. There were more patients with severe cGVHD who received sirolimus (Table 1), but there was no association between the use of sirolimus and telomerase activity of Tcon ($P = .71$) or Treg ($P = .22$), suggesting that telomerase activity was not affected by sirolimus. Although telomere length in Treg was similar in all patient subsets, Treg in all patients have very short telomeres compared with healthy subjects, and the observation that patients with moderate and severe cGVHD have very low telomerase activity suggests that this subset may be highly susceptible to premature senescence and shortened survival. In contrast, relatively high levels of Treg telomerase in patients with no or mild cGVHD suggests that these cells are less susceptible to premature senescence and may help explain the ability of these patients to maintain significantly higher numbers of Treg in vivo despite high rates of proliferation (Figure 4) in all patient subsets.

Although the primary effect of telomerase activity is to maintain telomere length in dividing cells, there have been several reports linking activation of telomerase to other pathways that function coordinately to enhance cell survival.[34] For example, previous studies have reported that inhibition of telomerase activity for short periods was associated with increased vulnerability to apoptosis without concomitant telomere shortening; and conversely, cells with increased telomerase activity exhibit reduced sensitivity to drug-induced apoptosis.[35-37] Studies have also reported that ectopic expression of human telomerase reverse transcriptase in T cells was associated with increased expression of Bcl-2, an antiapoptotic protein.[38] To examine whether telomerase activity of Tcon and Treg after HSCT was associated with Bcl-2 expression, we measured expression of this intracellular protein by flow cytometry in all patient samples. Bcl-2 expression was greater in Tcon than in Treg, but overall levels of expression were similar in patients with cGVHD and healthy subjects (Figure 5). Bcl-2 expression in Tcon was similar in all patient groups, but Bcl-2 expression in Treg was significantly higher in patients with no or mild cGVHD and lower in patients with moderate and severe cGVHD. When both Bcl-2 expression and telomerase activity were examined, there was a highly significant positive correlation in Treg but not in Tcon. Although the mechanisms responsible for this close association are not known, both of these pathways appear to function in a coordinated way to enhance Treg survival in some patients and

increase susceptibility to apoptosis in other patients. These functional effects are closely associated with the severity of cGVHD, providing further evidence that activation of the telomerase pathway in Treg contributes to the maintenance of peripheral tolerance and control of alloreactive cells after HSCT.

Both Treg and Tcon undergo homeostatic proliferation and expansion after HSCT, and our studies demonstrate that proliferative activity of Tcon remains significantly elevated in patients > 3.5 years after HSCT compared with healthy subjects. However, the magnitude of this difference is relatively small and the proliferative activity of Tcon is much less than Treg, even in patients with moderate or severe cGVHD. Consistent with this small increase in Tcon proliferation in patients with cGVHD, there was no evidence of telomerase activation, and levels of Tcon telomerase activity were actually lower than in healthy subjects. In contrast to Treg that remain highly proliferative for prolonged periods after transplantation, telomerase does not appear to play an important role in modulating Tcon survival and preventing premature senescence, even in patients with active cGVHD.

In conclusion, telomerase appears to play an important role in promoting survival and preventing premature senescence of Treg after allogeneic HSCT. In part, telomerase function is critical in these cells because maintenance of the Treg population is largely dependent on extensive proliferation and continuous expansion of mature Treg that is maintained for prolonged periods. Thus, patients in whom telomerase activity in Treg is maintained at high levels appear to be able to maintain higher numbers of circulating Treg. This probably contributes to suppression of cGVHD and maintenance of peripheral tolerance. In contrast, patients in whom telomerase activity in Treg is very low do not appear to be able to maintain adequate numbers of Treg and are not able to prevent the development of severe cGVHD after HSCT. Interestingly, telomerase activation appears to promote resistance to apoptosis through mechanisms that are associated with increased expression of Bcl-2 as well as through direct prevention of telomere shortening. The mechanisms responsible for differential regulation of telomerase activity of Treg and Tcon after HSCT remain to be established. These mechanisms may also include other antiapoptotic proteins, such as Bcl-XL or Mcl-1, as well as various cytokines associated with T-cell homeostasis. Telomerase is a holoenzyme protein regulated by several transcriptional and post-translational mechanisms. Transcription factors, such as myc, Sp-1, NF-κB protein, and nuclear factor of activated T cells, can bind to the promoter site of human telomerase reverse transcriptase DNA and enhance gene transcription.[39-41] Protein phosphorylation and chaperone proteins are also essential for maintenance of telomerase structure and function.[42,43] When the complicated regulation of Treg telomerase activity is elucidated and the molecules that selectively enhance telomerase activity of Treg can be defined, it may become possible to modulate this process in vivo. This may result in the ability to selectively promote the survival and expansion of Treg and the development of new methods to maintain immune tolerance. Our studies suggest that modulation of telomerase may provide an entirely new approach for prevention and treatment of cGVHD after allogeneic HSCT.

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Authorship

Contribution: Y.K. designed and performed the research, analyzed and interpreted the data, and wrote the manuscript; H.T.K. performed statistical analysis and participated in writing the paper; K.M. performed the research and analyzed the data; G.B. and S.M. analyzed patient and healthy subject samples; V.T.H., C.C., J.K., E.P.A., J.H.A., and R.J.S. provided vital patient samples and clinical information and edited the paper; and J.R. supervised the work, contributed to design, and interpretation of the study and edited the paper.

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References

1. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med.* 2010;363(22):2091-2101.
2. Arora M, Nagaraj S, Witte J, et al. New classification of chronic GVHD: added clarity from the consensus diagnoses. *Bone Marrow Transplant.* 2009;43(2):149-153.
3. Pavletic S, Vogelsand GB. Treatment of high-risk chronic GVHD. *Biol Blood Marrow Transplant.* 2008;14(12):1436-1437.
4. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* 2008;133(5):775-787.
5. Kriegel MA, Lohmann T, Gabler C, Blank N, Kalden JR, Lorenz HM. Defective suppressor function of human CD4+ CD25+ regulatory T cells in autoimmune polyglandular syndrome type II. *J Exp Med.* 2004;199(9):1285-1291.
6. Miyara M, Amoura Z, Parizot C, et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J Immunol.* 2005;175(12):8392-8400.
7. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med.* 2004;199(7):971-979.
8. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med.* 2004;10(9):942-949.
9. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood.* 2002;99(10):3493-3499.
10. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med.* 2002;196(3):401-406.
11. Johnson BD, Konkol MC, Truitt RL. CD25+ immunoregulatory T-cells of donor origin suppress alloreactivity after BMT. *Biol Blood Marrow Transplant.* 2002;8(10):525-535.
12. Zorn E, Kim HT, Lee SJ, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood.* 2005;106(8):2903-2911.
13. Li Q, Zhai Z, Xu X, et al. Decrease of CD4(+)CD25(+) regulatory T cells and TGF-beta at early immune reconstitution is associated to the onset and severity of graft-versus-host disease following allogeneic haematogenesis stem cell transplantation. *Leuk Res.* 2010;34(9):1158-1168.
14. Matsuoka K, Kim HT, McDonough S, et al. Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation. *J Clin Invest.* 2010;120(5):1479-1493.
15. Watson JD. Origin of concatemeric T7 DNA. *Nat New Biol.* 1972;239(94):197-201.
16. Olovnikov AM. A theory of marginotomy: the incomplete copying of template margin in enzymic

synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol.* 1973; 41(1):181-190.

17. Artandi SE, Chang S, Lee SL, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature.* 2000;406(6796):641-645.
18. Bailey SM, Murnane JP. Telomeres, chromosome instability and cancer. *Nucleic Acids Res.* 2006; 34(8):2408-2417.
19. Murnane JP. Telomeres and chromosome instability. *DNA Repair (Amst).* 2006;5(9):1082-1092.
20. Cheung AL, Deng W. Telomere dysfunction, genome instability and cancer. *Front Biosci.* 2008; 13:2075-2090.
21. Calado RT, Young NS. Telomere diseases. *N Engl J Med.* 2009;361(24):2353-2365.
22. Weng NP, Levine BL, June CH, Hodes RJ. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J Exp Med.* 1996;183(6):2471-2479.
23. Bodnar AG, Kim NW, Effros RB, Chiu CP. Mechanism of telomerase induction during T cell activation. *Exp Cell Res.* 1996;228(1):58-64.
24. Akiyama M, Hoshi Y, Sakurai S, Yamada H, Yamada O, Mizoguchi H. Changes of telomere length in children after hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 1998; 21(2):167-171.
25. Akiyama M, Asai O, Kuraishi Y, et al. Shortening of telomeres in recipients of both autologous and allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 2000;25(4):441-447.
26. Baerlocher GM, Rovo A, Muller A, et al. Cellular senescence of white blood cells in very long-term survivors after allogeneic hematopoietic stem cell transplantation: the role of chronic graft-versus-host disease and female donor sex. *Blood.* 2009; 114(1):219-222.
27. Vukmanovic-Stejic M, Zhang Y, Cook JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest.* 2006;116(9):2423-2433.
28. Wolf D, Rumpold H, Koppelstatter C, et al. Telomere length of in vivo expanded CD4(+)CD25 (+) regulatory T-cells is preserved in cancer patients. *Cancer Immunol Immunother.* 2006; 55(10):1198-1208.
29. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant.* 2005;11(12):945-956.
30. McGrath M, Wong JY, Michaud D, Hunter DJ, De Vivo I. Telomere length, cigarette smoking, and bladder cancer risk in men and women. *Cancer Epidemiol Biomarkers Prev.* 2007;16(4):815-819.
31. Emrich T, Karl G. Nonradioactive detection of telomerase activity using a PCR-ELISA-based telomeric repeat amplification protocol. *Methods Mol Biol.* 2002;191:147-158.
32. Wolf D, Wolf AM, Fong D, et al. Regulatory T-cells in the graft and the risk of acute graft-versus-host disease after allogeneic stem cell transplantation. *Transplantation.* 2007;83(8):1107-1113.
33. Weng NP, Granger L, Hodes RJ. Telomere lengthening and telomerase activation during human B cell differentiation. *Proc Natl Acad Sci U S A.* 1997;94(20):10827-10832.
34. Nitta E, Yamashita M, Hosokawa K, et al. Telomerase reverse transcriptase protects ATM-deficient hematopoietic stem cells from ROS-induced apoptosis through a telomere-independent mechanism. *Blood.* 2011;117(16):4169-4180.
35. Kondo Y, Kondo S, Tanaka Y, Haqqi T, Barna BP, Cowell JK. Inhibition of telomerase increases the susceptibility of human malignant glioblastoma cells to cisplatin-induced apoptosis. *Oncogene.* 1998;16(17):2243-2248.
36. Massard C, Zermati Y, Pauleau AL, et al. hTERT: a novel endogenous inhibitor of the mitochondrial cell death pathway. *Oncogene.* 2006;25(33): 4505-4514.
37. Kaul D, Gautam A, Varma S, Ahlawat A. Functional genomics of hTERT gene in leukemic myelopoiesis. *Mol Cell Biochem.* 2008;314(1):19-23.
38. Luiten RM, Pene J, Yssel H, Spits H. Ectopic hTERT expression extends the life span of human CD4+ helper and regulatory T-cell clones and confers resistance to oxidative stress-induced apoptosis. *Blood.* 2003;101(11):4512-4519.
39. Chebel A, Rouault JP, Urbanowicz I, et al. Transcriptional activation of hTERT, the human telomerase reverse transcriptase, by nuclear factor of activated T cells. *J Biol Chem.* 2009;284(51): 35725-35734.
40. Kyo S, Takakura M, Taira T, et al. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res.* 2000;28(3):669-677.
41. Sinha-Datta U, Horikawa I, Michishita E, et al. Transcriptional activation of hTERT through the NF-kappaB pathway in HTLV-I-transformed cells. *Blood.* 2004;104(8):2523-2531.
42. Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem.* 1999;274(19): 13085-13090.
43. Forsythe HL, Jarvis JL, Turner JW, Elmore LW, Holt SE. Stable association of hsp90 and p23, but Not hsp70, with active human telomerase. *J Biol Chem.* 2001;276(19):15571-15574.