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Altered Homeostasis of CD4$^+$ Memory T Cells in Allogeneic Hematopoietic Stem Cell Transplant Recipients: Chronic Graft-versus-Host Disease Enhances T Cell Differentiation and Exhausts Central Memory T Cell Pool

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ABSTRACT

An increased risk of late infection is a serious complication after allogeneic hematopoietic stem cell transplantation (AHSCT), especially for recipients with defective CD4$^+$ T cell recovery. Although chronic graft-versus-host disease (cGVHD) negatively influences CD4$^+$ T cell reconstitution, the mechanisms leading to this defect are not well understood. We found that the proportion of CD27$^-$ CD4$^+$ T cells was remarkably increased in ASHCT recipients with cGVHD or with repetitive infectious episodes. Isolated CD27$^-$ CD4$^+$ T cells from ASHCT recipients had significantly shortened telomere length, displayed enhanced vulnerability to activation-induced cell death, and showed extremely reduced clonal diversity, when compared with CD27$^-$ CD4$^+$ T cells from healthy donors. Also, CD27$^+$ CD4$^+$ T cells from AHSCT recipients easily lost their expression of CD27 in response to antigen stimulation regardless of cGVHD status. Taken together, these data indicate that homeostasis of memory CD4$^+$ T cells from AHSCT recipients is altered, and that they easily transit into CD27$^-$ effector memory T cells. Increased in vivo T cell stimulation observed in recipients with cGVHD further promotes the transition to effector memory cells, a change that decreases the central memory CD4$^+$ T cell pool and consequently weakens the recipient's defense against persistently infecting pathogens.

KEY WORDS

Chronic GVHD • CD27 • T cell memory • T cell reconstitution

INTRODUCTION

An increased risk of fatal infection remains the major problem for long-term survivors of allogeneic hematopoietic stem cell transplantation (AHSCT). After the immediate posttransplant period, AHSCT recipients experience long-lasting immune deficiency [1]. Several studies have demonstrated that quantitative and qualitative recovery of CD4$^+$ T cells both play pivotal roles in protecting against late infections and in improving the long-term outcome of AHSCT recipients [2-4]. T cell reconstitution can involve both a thymus-dependent pathway and a thymus-independent pathway. In the early posttransplant period, a thymus-independent peripheral expansion of memory T cells occurs [5]. Because the reconstitution of the CD4$^+$ T cell pool is more dependent on thymic function [6], CD8$^+$ T cells predominate over CD4$^+$ T cells for several months after AHSCT [5]. In the setting of myeloablative autologous transplantation in adults, it is reported that CD4$^+$ T cell regeneration and thymic enlargement occur by 6 months, and the number and diversity of naïve CD4$^+$ T cells normalizes 2 years after transplantation, at least in nonelderly patients [7]. After AHSCT, reconstitution of CD4$^+$ T cells is influenced not only by recipient age, but also

by sources of stem cells and, most important, by graft-versus-host disease (GVHD) [6,8-11].

Chronic GVHD (cGVHD) is a vexing late complication that deeply influences the long-term outcomes of AHSCT recipients. The disease develops in about 50% of AHSCT survivors, and for many is resolved within 2 years following AHSCT [12-15]. Unfortunately, some patients experience refractory disease for an extended period, and their probability of long-term survival is lowered by opportunistic infection [12,16,17]. The presence of active cGVHD negatively influence the recovery of $CD4^+$ T cells [11]; however, how cGVHD impairs the functional recovery of $CD4^+$ T cells is not yet well understood.

To clarify this mechanism, we analyzed the characteristics of peripheral blood $CD4^+$ T cells in patients who received AHSCT at least 1 year earlier. We also aimed to establish reliable clinical indicators for use in identifying AHSCT recipients for whom reinforced protection against late infection would be highly recommended.

PATIENTS, MATERIALS, AND METHODS

Patients and Samples

Blood samples were obtained from 40 AHSCT recipients and 15 healthy donors after receiving their written informed consents. AHSCT recipients had undergone transplantation 1 to 10 years prior. All patients were confirmed to be in complete donor chimerism and in complete remission. To exclude the effects on T cell characteristics derived from any concurrent infectious episodes, blood samples from febrile patients were not drawn. The clinical characteristics of AHSCT recipients are summarized in Table 1. The presence of cGVHD was defined as follows: dependency on immunosuppressive agents in patients who had received transplantation >3 years before, or the presence of active symptoms for which immunosuppressive therapy was required in patients receiving transplantations 1 to 3 years earlier. All studies using these blood samples were approved by the Medical Ethics Committee of Kyoto University.

Table 1. *Characteristics of Patients, Donors, and Transplantations*

Parameter	Values*
Sex of patients	
Male/female	21/19
Median age of patients (range)	
Patients	47 (20-70)
Donors**	41 (20-63)
Donor type	
MSD/Non-MSD	15/25
Stem cell source	
Bone marrow	28
Peripheral blood	10
Cord blood	2
Diagnosis	
Acute leukemia	9
Chronic myeloproliferative disease	8
Lymphoma or myeloma	13
Myelodysplastic syndrome	5
Aplastic Anemia	4
Adrenoleukodystrophy	1
Conditioning regimen	
Myeloablative	25
Reduced intensity	15
GVHD prophylaxis	
Cyclosporine-based	9
Tacrolimus-based	31
Interval after AHSCT	
1 to 3 years	18
3 to 5 years	15
more than 5 years	7

MSD indicates matched sibling donor; GVHD, graft-versus-host disease; AHSCT, allogeneic hematopoietic stem cell transplantation.
*Values are expressed as number of patients otherwise indicated.
**Cord blood donor is excluded.

Monoclonal Antibodies and Flow Cytometry

Mononuclear cells from the peripheral blood of AHSCT recipients or healthy donors (HDs) were separated by Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient sedimentation. For analysis of cell surface antigens, cells were incubated with an appropriate concentration of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-cyanin-5 (PC5)-, or allophycocyanin (APC)-conjugated monoclonal antibody (mAb) or the corresponding isotype-matched control mAb (eBiosience, San Diego, CA) in the dark at 4°C for 20 minutes. They were then washed twice and analyzed by FACScalibur (BD Biosciences, San Jose, CA) with CellQuest software (BD Biosciences). MAbs used in this study included FITC-conjugated anti-CD27 (FITC-CD27), FITC-HLA-DR, FITC-CD57, PE-CD27, PE-CCR7, FITC-CD62L, APC- CD45RA (BD PharMingen, San Jose, CA), and PC5-CD4 (Beckman Coulter, Fullerton, CA). FITC- and PE-conjugated mAbs were purchased from eBioscience. For intracellular cytokine staining, cells were suspended in culture medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 10% volume fetal calf serum (FCS, Hyclone, Logan, UT), and antibiotics. They were then stimulated with immobilized anti-CD3 mAb (OKT3, 10 μg/mL) and 2 μg/mL soluble anti-CD28 mAb (clone48, agonistic antibody established in our laboratory; T. Hori, unpublished data) for 5 hours in the presence of 10 μg/mL brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO). After cell surface staining, cells were fixed with 2% formaldehyde (Wako Pure Chemical Industries, Osaka, Japan) diluted in phosphate-buffered saline (PBS), and permeabilized with 0.2% saponin (Sigma-Aldrich) containing buffer. After washing with saponin-containing buffer, cells were incubated with FITC-conjugated anti-interferon-γ

(IFN-γ), FITC-conjugated anti-interleukin-2 (IL-2), or FITC-conjugated isotype-matched control mAb. All mAbs used for cytokine staining were obtained from BD PharMingen.

$CD4^+$ T Cell Separation and Cell Sorting

$CD4^+$ T cells were isolated by a MACS CD4 multisort kit (Miltenyi Biotec, Bergisch Gladbach, Germany), using MACS separation MS columns (Miltenyi Biotec) according to the manufacturer's recommendations. They were then stained with PE-CD27 and APC-CD45RA and sorted into naïve ($CD27^+$/$CD45RA^+$), $CD27^+$ memory ($CD27^+$/$CD45RA^-$), and $CD27^-$ memory cells using FACSAria (BD Biosciences) with FACSDiVa 4.1 software (BD Biosciences). In some experiments, $CD4^+$ T cells from AHSCT recipients were stained with PE-CD27 only and sorted into $CD27^+$ and $CD27^-$ cells. Sorted T cell subsets displayed a purity of at least 98%.

T Cell Stimulation and Short-Term Culture

Isolated naïve, $CD27^+$ memory, and $CD27^-$ memory T cells were suspended in a culture medium at a concentration of 5×10^5/mL, and 1 mL of cell suspension was transferred into a 48-well plate coated with anti-CD3 mAb. The concentration of anti-CD3 mAb was either high (10 μg/mL) or low (0.1 μg/mL). Anti-CD28 mAb (2 μg/mL) was also added to the culture. T cell stimulation with anti-CD3 and anti-CD28 mAbs (CD3/28 stimulation) was performed for 3 days; the cells were then harvested, washed twice, and incubated with culture medium supplemented with 150 IU/mL IL-2 (Peprotec, Rocky Hill, NJ) at a concentration of 2×10^5/mL for an additional 4 days. In some experiments, after 7-day cultivation, the cells were harvested, washed, prepared at a concentration of 5×10^5/mL in culture medium, and restimulated overnight on an anti-CD3 mAb-coated plate (10μg/mL). T cell death was determined by propidium iodide (PI, Sigma-Aldrich) staining in which a final concentration of 1 μg/mL of PI was added to each cell sample before flow cytometry analysis.

Flow-FISH

Relative telomere length (RTL) measurements of $CD27^+$ memory and $CD27^-$ memory $CD4^+$ T cells were performed by a flow-FISH method using a Dako Telomere peptide nucleic acid (PNA) kit/FITC (Dako, Glostrup, Denmark), according to the manufacturer's instructions. In brief, isolated cells and control cells (1301 cell line; Interlab Cell Line Collection, Genoa, Italy) were mixed in equal measure in hybridization solution with or without FITC-labeled telomere PNA probe for 10 minutes at 82°C; hybridized overnight in the dark at room temperature; washed twice with a wash solution at 40°C; resuspended in PBS containing 2% FCS and PI (1 μg/mL); and subjected to flow cytometric analysis. Telomere fluorescence of at least 100,000 events was measured at a low flow rate to ensure low coefficients of variation. The RTL value was calculated using the following formula: RTL = ([mean FL1 sample cells with probe − mean FL1 sample cells without probe] × DNA index of control cells) ÷ ([mean FL1 control cells with probe − mean FL1 control cells without probe] × DNA index of sample cells) × 100. Because 1301 is a tetraploid cell line, the DNA index of 1301 cells is 2 and the DNA index of sample cells in this study is 1.

T Cell Receptor Repertoire Analysis and Complementarity-Determining Region 3 Size Spectratyping

RNA isolated from naïve, $CD27^+$ memory, and $CD27^-$ memory cells was converted to double-stranded complementary DNA (cDNA), and T cell receptor (TCR) beta chain variable region (VB) repertoires were analyzed with an adaptor ligation polymerase chain reaction (PCR)-based microplate hybridization assay [18]. After PCR amplifications using primers specific for each TCRVB, the 5′-biotinylated PCR products were hybridized in microplate wells on which 38 TCRVB-specific probes were immobilized, and followed with quantitative ELISA. PCR products were also subjected to analysis for complementary-determining region 3 (CDR3) size spectratyping [19]; they were diluted in dye solution (95% formamide, 10 mM EDTA, and 0.1% blue dextrane) and analyzed in 6% denatured acrylamide gel with an ALFred sequence analyzer (Pharmacia Biotech, Uppsala, Sweden). The data obtained were transferred to Fragment manager software (Pharmacia Biotech).

Statistical Analysis

We used a 2-sided Mann-Whitney *U* test (nonpaired samples) and a paired *t*-test (paired samples) for an analysis of differences between the groups. *P* values $<.05$ were considered statistically significant.

RESULTS

The Proportion of $CD27^-$ $CD4^+$ T Cells Increased in AHSCT Recipients, Especially in Those with cGVHD

During comparative analysis of peripheral $CD4^+$ T cells from AHSCT recipients and HDs, we found that the proportion of $CD27^-$ cells among total $CD4^+$ T cells was substantially higher in AHSCT recipients who had been transplanted more than 1 year previously. As shown in Figure 1A, identified 3 major subsets of $CD4^+$ T cells by dual staining, using CD45RA and CD27. The $CD45RA^+$/$CD27^+$ fraction represents naïve T cells, and the $CD27^-$ fraction

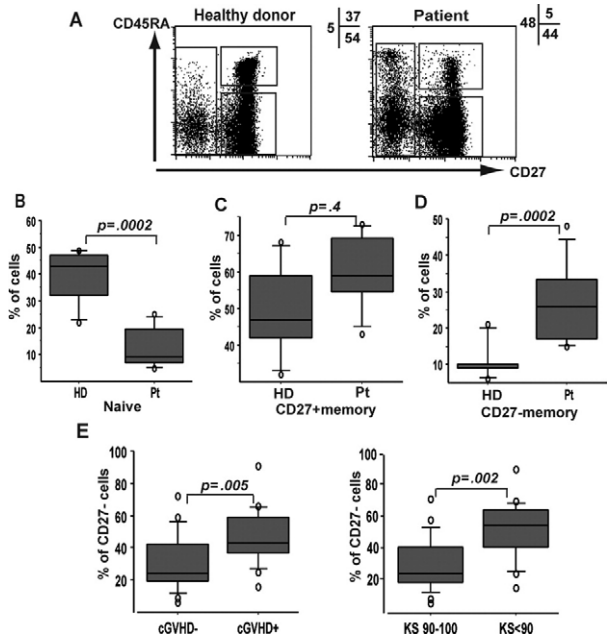

Figure 1. Proportion of CD27$^-$ cells among total CD4$^+$ T cells in AHSCT recipients and healthy donors. Peripheral lymphocytes from AHSCT recipients and HDs were stained with CD4, CD27, and CD45RA and gates were set on CD4-positive lymphocytes. A, Representative dot plots of CD45RA and CD27 are shown. The percentage of CD27$^+$ CD45RA$^+$ naïve, CD27$^+$ CD45RA$^-$ memory, and CD27$^-$ memory cells are shown in the upper right of each plot. The percentage of naïve (B), CD27$^+$ memory (C), and CD27$^-$ memory cells (D) among total CD4$^+$ T cells was compared between 16 AHSCT recipients and 15 HDs. E, Comparison of the percentage of CD27$^-$ memory cells among total CD4$^+$ T cells between AHSCT recipients in the presence or absence of cGVHD (left), and with or without good physical condition (right). Samples from 40 AHSCT recipients were included. The median, 25th-75th percentiles, 10th-90th percentiles, and outliers are represented by the line in the box, shaded box, error bars, and open circles, respectively. A Mann-Whitney *U* test was used for statistical analysis.

is known to include terminally differentiated cells [20]. The CD45RA$^-$/CD27$^+$ fraction is composed of central memory T cells [21]. When data from 19 AHSCT recipients (interval after AHSCT ranged from 1.5 to 10 years, median 4 years) and 15 HDs were compared, it was clear that the percentage of naïve T cells was low, and that of CD27$^-$ memory cells was substantially high within the CD4$^+$ T cell subset from AHSCT recipients (Figure 1B-D). To search for a correlation between the percentage of CD27$^-$ cells among total CD4$^+$ T cells and the clinical parameters, samples from an additional 21 patients who had received AHSCT more than 1 year before were collected. Statistical analysis revealed 2 variables associated with the increased proportion of the CD27$^-$ fraction: the presence of cGVHD and a poor general condition, defined by a Karnofsky score of less than 90 (Figure 1E). An analysis of clinical charts demonstrated that repetitive infectious episodes reduced the general condition of most patients, and many of them (but not all) also suffered from cGVHD (data not shown). The frequency of CD27$^-$ cells among CD4$^+$ T cells did not correlate with recipient age, source of stem cells, donor type, underlying disease, conditioning regimen, history of acute GVHD (aGVHD), or interval after transplantation (data not shown). Although van Leeuwen reported that individuals who were persistently infected with cytomegalovirus (CMV) showed increased frequency of CD27$^-$ CD28$^-$ cells within CD4$^+$ T cells [22], in our experiments, no pair of AHSCT recipient and donor was found to be serologically negative for CMV at the time of SCT; thus, we could not evaluate the correlation between persistent CMV infection and the proportion of CD27$^-$ cells.

We then analyzed surface phenotype and cytokine profiles of CD27$^-$ CD4$^+$ T cells from AHSCT recipients. Multicolor flow cytometry revealed that CD27$^-$ CD4$^+$ T cells preferentially expressed HLA-DR and CD57 and expressed very little CCR7 or CD62L (data not shown). Cytokine profiles of CD27$^-$ CD4$^+$ T cells were determined by sorting CD4$^+$ T cells into CD27$^+$ and CD27$^-$ fractions. CD3/28 stimulation followed by intracytoplasmic cytokine-staining revealed that CD27$^-$ cells produced IFN-γ much more frequently than CD27$^+$ cells, and the proportion of IL-2-producing cells was comparable (data not shown). The expression of surface molecules and cytokine-producing capacity showed no significant difference between CD27$^-$ CD4$^+$ T cells from AHSCT recipients and HDs (data not shown). These results are consistent with previous studies and demonstrating that CD27$^-$ CD4$^+$ T cells are differentiated effector cells [21,23].

Shortened Telomere Length and Enhanced Vulnerability to Activation-Induced Cell Death of CD27$^-$ CD4$^+$ T Cells from AHSCT Recipients

To better understand the differentiated status of CD27$^-$ cells in AHSCT recipients, we examined and compared the telomere length of sorted CD4$^+$ T cell fractions derived from AHSCT recipients and HDs. CD4$^+$ T cells from HDs were sorted into naïve, CD27$^+$ memory, and CD27$^-$ memory cells as shown in Figure 2A. In CD4$^+$ T cells from AHSCT recipients, naïve cells were not separated from CD27$^+$ memory cells because of the limited total cell number available and the scarcity of naïve cells. Figure 2B shows representative dot plots for the measurements of relative telomere lengths (RTLs) of sample cells, using the flow-FISH method. The RTL of CD27$^-$ cells was shorter when compared with those of CD27$^+$ cells in AHSCT recipients and HDs (Figure 2C). Most important, CD27$^-$ cells from AHSCT recipients showed significantly shorter RTLs compared with those from HDs (Figure 2C). Because previous

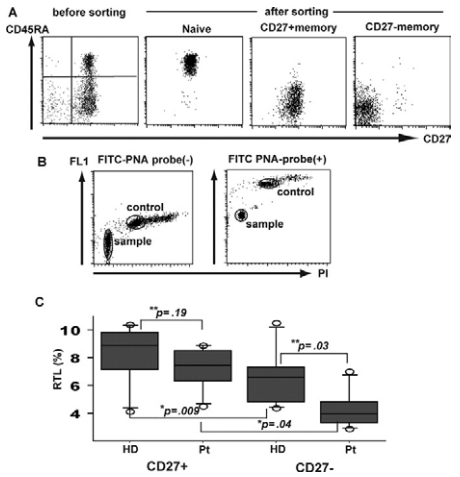

Figure 2. Shortened telomere length in CD27$^-$ CD4$^+$ T cells. Magnetically isolated, peripheral blood CD4$^+$ T cells from AHSCT recipients were stained with CD27 and sorted into CD27$^+$ and CD27$^-$ cells. CD4$^+$ T cells from HDs were stained with CD27 and CD45RA and sorted into CD27$^+$ memory and CD27$^-$ memory T cells. A, Representative dot plots of HD-derived CD4$^+$ T cells before and after cell sorting are shown. B, Sorted T cells were individually mixed in equal measure with control cell line 1301, hybridized with or without an FITC-labeled PNA-telomere probe, counterstained with PI, and analyzed by FACScalibur. The percent RTL (relative telomere length) was calculated as described in the Patients, Materials, and Methods section. C, The percent of RTL in CD27$^+$ and CD27$^-$ cells from 6 AHSCT recipients and in CD27$^+$ memory and CD27$^-$ memory cells from 6 HDs were analyzed by flow-FISH. Statistical analysis was performed using a paired *t*-test (*) and Mann-Whitney *U* test (**).

work demonstrated that telomere length shortens in the course of T cell differentiation [24], these results indicated that CD27$^-$ CD4$^+$ T cells from AHSCT recipients undergo more cell divisions and are more differentiated than those from HDs.

We next evaluated the vulnerability to activation-induced cell death (AICD), because differentiated T cells are known to be susceptible to apoptosis [25]. Sorted CD27$^-$ and CD27$^+$ CD4$^+$ T cells from 8 AHSCT recipients (4 of them complaining of cGVHD) and CD27$^-$ and CD27$^+$ memory CD4$^+$ T cells from 9 HDs were subjected to CD3/28 stimulation, cultivation with IL-2, and restimulation with anti-CD3 mAb [26]. As expected in every patient and healthy donor, CD27$^-$ CD4$^+$ T cells were more susceptible to IL-2-induced AICD after a second stimulation than were CD27$^+$ CD4$^+$ T cells (Figure 3A and B). In accordance with a shorter telomere length, CD27$^-$ CD4$^+$ T cells from AHSCT recipients showed more cell deaths than those from HDs. CD27$^+$ CD4$^+$ T cells from AHSCT recipients showed comparable vulnerability to AICD whether from patients with or without cGVHD (Figure 3B).

Substantially Reduced T Cell Repertoire Diversity of CD27$^-$ CD4$^+$ T Cells from AHSCT Recipients

For effective immunity against various pathogens, broad T cell repertoire diversity is essential [27]. Reported experiments using total lymphocytes or sorted CD4$^+$ T cells have shown that skewing of T cell repertoire after stem cell transplantation continues for an extended period [28,29]. To better understand immunologic reconstitution after AHSCT, sorted naïve, CD27$^+$ memory, and CD27$^-$ memory CD4$^+$ T cells were individually subjected to microplate hybridization assay to determine TCRVB repertoires, and perform CDR3 spectratyping. As shown in Figure 4, we found that naïve cells and CD27$^+$ memory cells from AHSCT recipients showed diverse CDR3 size spectratypes with a Gaussian distribution of TCR fragments, identical to those from HDs [30]. In contrast, the TCRVB repertoire of CD27$^-$ memory CD4$^+$ T cells was prominently skewed, and CDR3 size spectratypes were characterized by oligoclonal expansions, which was much more prominent in AHSCT recipients.

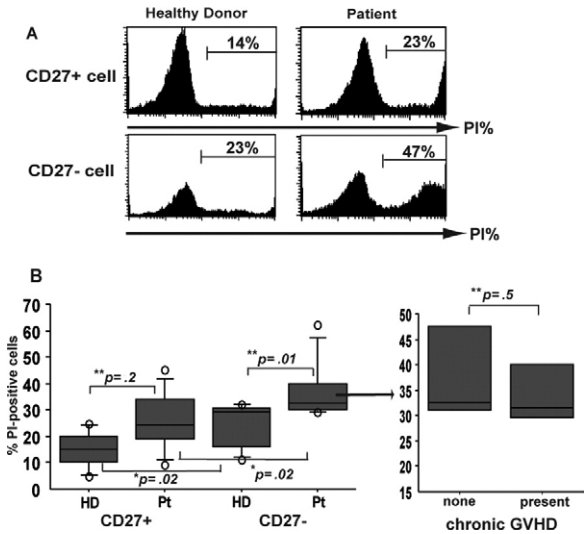

Figure 3. IL-2-induced AICD assay of CD4$^+$ T cells from AHSCT recipients and HDs. Isolated CD4$^+$ T cells from AHSCT recipients were sorted into CD27$^+$ and CD27$^-$ T cells. Memory CD4$^+$ T cells from HDs were sorted into CD27$^+$ memory, and CD27$^-$ memory cells. Sorted cells were stimulated with 0.1 μg/mL plate-bound anti-CD3 mAb and 2 μg/mL soluble anti-CD28 mAb for 3 days. Cells were then cultured with IL-2 for another 4 days and restimulated with 10 μg/mL plate bound anti-CD3 mAb overnight. The collected cells were stained with PI and the ratio of PI-positive cells was analyzed by flow cytometry. A, Representative histogram of PI staining. Values shown denote the percentage of nonviable cells. B, The vulnerability to IL-2-induced AICD was compared between CD27$^+$ and CD27$^-$ cells from 8 AHSCT recipients and 9 HDs (left). A comparison of the ratio of PI-positive cells among CD27$^-$ T cells from AHSCT recipients with or without cGVHD is shown (right). Statistical analysis was performed using a paired *t*-test (*) and a Mann-Whitney *U* test (**).

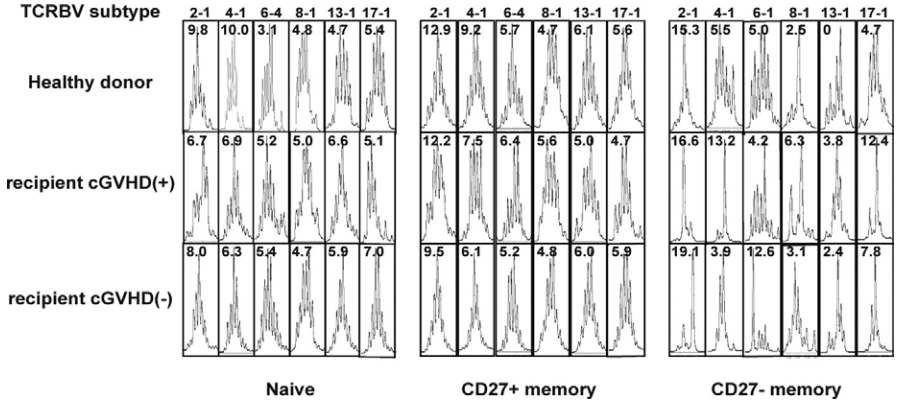

Figure 4. TCR repertoire analysis of sorted CD4$^+$ T cells. RNA was isolated from sorted naïve, CD27$^+$ memory, and CD27$^+$ memory CD4$^+$ T cells from AHSCT recipients as well as from HDs. TCR repertoire analysis, followed by CDR3 size spectratyping, was performed as described in the Patients, Materials, and Methods section. Because the frequency of TCRVB 2-1, 4-1, 6-4, 8-1, 13-1, and 17-1 was dominated in most individuals examined, CDR3 spectratypes of these TCRVBs are shown. Values in each spectratype represent the frequency of individual TCRVBs among a sorted population defined by microplate hybridazation assay. The data are representative of 3 HDs and 5 AHSCT recipients.

CD27$^+$ CD4$^+$ T Cells from AHSCT Recipients Easily Lost CD27 Molecules

We next investigated the mechanism by which the percentage of peripheral CD27$^-$ CD4$^+$ T cells increases in AHSCT recipients. We hypothesized that transition of CD27$^+$ memory T cells into CD27$^-$ cells was accelerated to cause this effect. As shown previously, CD27$^+$ memory cells are composed of CCR7$^+$ "central memory" and CCR7$^-$ "effector memory" subpopulations [21,31]. We found that the frequency of CCR7$^+$ cells in CD27$^+$ memory cells was similar between AHSCT recipients and HDs (data not shown), indicating that CD27$^+$ memory cells from AHSCT recipients were, as a whole, not significantly different in differentiation than those from HDs. The kinetics of CD27 expression in response to CD3/28 stimulation was examined with sorted CD27$^+$ CD4$^+$ T cells. Naïve cells from HDs showed negligible loss of CD27 expression upon stimulation in either strong (plate-bound OKT3 at 10 μg/mL) or weak (0.1 μg/mL) conditions. Strong but not weak stimulation conditions decreased the percentage of CD27$^+$ cells in CD27$^+$ memory T cells from those of HDs. In contrast, CD27$^+$ cells from AHSCT recipients, which included a minor population of naïve cells, remarkably lost their CD27 expression upon both strong and weak stimulation (Figure 5A). The presence or absence of cGVHD did not affect the extent of downregulation of CD27 expression.

DISCUSSION

Recent studies indicate that CD4$^+$ memory T cells can be divided into distinct populations of central memory and effector memory cells according to their CCR7 expression profiles [31]. Further, Fritsch proposed that effector memory T cells that lose expression of CD27 form a subpopulation of terminally differentiated memory cells [21]. In fact, prolonged stimulation of CD27$^+$ memory CD4$^+$ T cells was shown to result in irreversible loss of CD27 expression in vitro and in vivo [32,33]. CD27$^-$ CD4$^+$ T cells are enriched in inflamed peripheral tissues, such as the mucosal lamina propria of inflammatory bowel disease patients [34] or synovial tissues of rheumatoid arthritis patients [35]. The increase in CD27$^-$ memory cell frequency among peripheral total blood CD4$^+$ T cells has been demonstrated in patients with systemic lupus erythematosus [36] and in healthy elderly individuals [37], and the increase in the ratio of CD27$^-$ cells is assumed to be the consequence of either enhanced T cell differentiation induced by repetitive in vivo T cell stimulation [35,36] or of increased peripheral homeostatic expansion because of reduced thymic output [37,38].

In this study, we showed that the proportion of CD27$^-$ cells among total CD4$^+$ T cells, which is <10% in nonelderly healthy adults [20,39] and about 15% in patients with SLE [36] or in elderly individuals [37] was substantially increased for extended periods (up to 10 years) in AHSCT recipients (median 39.5%); it was especially high in patients with cGVHD (median 44.5%) or impaired general conditions resulting from repetitive infectious episodes (median 53.5%). Fallen and colleagues [40] also reported an increase in the number of CD27$^-$ CD4$^+$ T cells for patients within the first year after AHSCT when thymic function was not sufficiently recovered [9]. The CDR3 spectratypes of naïve and CD27$^+$ memory CD4$^+$ T cells clearly show that the thymic function of patients who received AHSCT >1 year

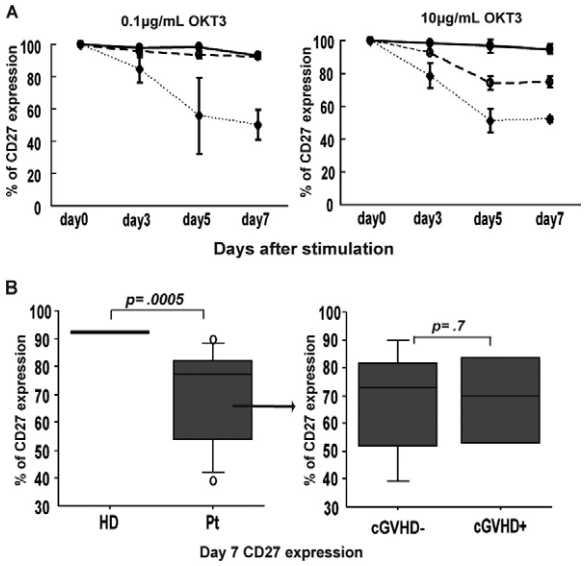

Figure 5. Kinetics of surface CD27 expression after CD3/28 stimulation. $CD27^+$ $CD4^+$ T cells obtained by cell sorting from AHSCT recipients, and naïve and $CD27^+$ memory $CD4^+$ T cells were isolated from HDs. The cells received CD3/28 stimulation for 3 days, followed by cultivation with IL-2 for 4 days. A, The cells were collected on days 3, 5, and 7, stained with FITC-CD27, and analyzed for CD27 expression with flow cytometry. The solid line and dotted line represents naïve cells, and $CD27^+$ memory T cells from HDs, respectively (N = 5). Fine dotted line indicates $CD27^+$ cells from AHSCT recipients (N = 4). In CD3/28 stimulation, weak (plate-bound anti-CD3 mAb, 0.1 μg/mL) and strong (10 μg/mL) conditions were utilized. B, (Left) The percentage of CD27-positive cells after a 7-day cultivation. $CD27^+$ cells from 9 AHSCT recipients and $CD27^+$ memory cells from 5 HDs were stimulated under weak conditions. (Right) Comparison of day 7 expression of CD27 between AHSCT recipients with or without cGVHD. Analysis was performed using the Mann-Whitney *U* test.

before is sufficient to maintain a T cell repertoire with normal diversity in terms of TCRVB usage; therefore, reduced thymic output does not fully explain the extremely elevated proportion of $CD27^-$ cells in AHSCT recipients.

In accordance with previous reports [21,23,35] we confirmed that $CD27^-$ $CD4^+$ T cells displayed more differentiation than $CD27^+$ memory cells. In addition, recent reports indicate that expression of CD57, which was more frequently detected on $CD27^-$ $CD4^+$ T cells in our study, correlates with replicative senescence [41,42]. Moreover, $CD27^-$ memory cells from AHSCT recipients showed greater reduction in telomere length and increased vulnerability to AICD than those from HDs. Several studies demonstrated that telomere length is useful in assessing the extent of differentiation in lymphocyte populations, and that T cells with shorter telomeres are accompanied by reduced telomerase induction, a diminished replicative capacity following activation, and an increased susceptibility to apoptosis [24,38]. The substantially reduced T cell repertoire diversity of $CD27^-$ cells from AHSCT recipients also supports the notion that these cells experience repetitive stimulation in vivo, resulting in a more prominent clonal expansion than the same cells in HDs. Because the proportion of $CD27^-$ cells greatly increases in patients with cGVHD, a major proportion of circulating $CD4^+$ T cells are derived from oligoclonal expansion of $CD27^-$ cells with reduced proliferative potentials.

$CD27^-$ $CD4^+$ T cells are cells involved in the first-line defense against pathogens. The combinatorial expression of adhesion molecules and chemokine receptors on $CD27^-$ $CD4^+$ T cells facilitates their immediate distribution into inflamed peripheral tissues, where they promptly produce effector cytokines and deliver direct cytotoxic effects [33,39,43]. Casazza et al. [33] reported that antigen-specific $CD4^+$ T cells, such as CMV-specific T cells, are abundant in this population. However, the significance of effector memory T cells in long-term immune control has recently become questionable. Heller et al. [44] demonstrated that protective immune control for persistently infecting pathogens require a reservoir of central memory T cells that continuously fuels effector memory cells. In addition, Fletcher et al. [45] reported that a large amount of CMV-specific T cells with a late differentiated phenotype ($CD27^-$, $CD28^-$) does not always contribute to effective immunity against CMV, because such cells showed severely restricted replicative capacity. An appropriate proportional balance between central memory and effector memory cells, between $CD27^+$ memory and $CD27^-$ memory $CD4^+$ T cells, is requisite for effective long-term control against persistently infecting pathogens, such as CMV.

We found that the ratio of $CD27^-$ cells significantly increases after delivery of CD3/28 stimulation to $CD27^+$ cells from AHSCT recipients when compared to those from HDs. As the condition of cGVHD did not affect the degree of downregulation, we suggest that $CD27^+$ cells from AHSCT recipients have an intrinsic tendency to easily differentiate into effector memory cells. Poulin et al. [10] demonstrated that the survival of naïve T cells in AHSCT recipients is shortened even in the absence of cGVHD. Our data suggested that the homeostasis of $CD27^+$ memory T cells is also altered in AHSCT recipients. In patients with cGVHD, sustained delivery of alloantigenic stimuli is presumed to drive alloreactive $CD27^+$ memory T cells to differentiate into $CD27^-$ cells. In addition, immune reaction associated with cGVHD could also promote the differentiation of nonhost-reactive $CD27^+$ memory T cells in a bystander fashion [45,46]. As an explanation for the correlation between increased frequency of $CD27^-$ $CD4^+$ T cells and repetitive infectious episodes, we posit the following rationale. In the course of infectious episodes, significant amount of $CD27^+$ central memory $CD4^+$

T cells receive differentiation-inducing stimuli in both an antigen-specific and bystander fashion. Because AHSCT recipients are at high risk of developing infections, especially when continuous use of corticosteroids is required, they have higher probability of suffering more than 1 infectious episode. As central memory T cells of AHSCT recipients easily differentiate into effector memory cells, repeated infections hasten the differentiation of $CD27^+$ memory cells, leaving the central memory T cell pool diminished. As a result, AHSCT recipients become more susceptible to opportunistic infections, and the $CD27^+$ memory T cell pool becomes exhausted.

Taken together, we propose a hypothesis for the defective immunity observed in AHSCT recipients: in vivo T cell stimulation in AHSCT recipients with cGVHD or repetitive infectious episodes profoundly promotes the differentiation of $CD27^+$ memory $CD4^+$ T cells; as a result, the $CD27^+$ memory cell pool decreases, which makes continuous control against persistently infecting pathogens difficult. We also propose that the ratio of $CD27^-$ cells among total $CD4^+$ T cells could serve as an effective clinical indicator, identifying patients who may likely require a more intensive regimen for protection against opportunistic infections.

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