Table 1.
Characteristics of Patients*

UPN	Age/Gender	Interval, m†	Red Cell Transfusion	Pretreatment Value‡		BM Central Review		Cytogenetics	IPSS Score	PNH-Type Cell	HLA-DR15 (DRB1)
				PLT	Neut	Cellularity	FAB/WHO				
1	18/M	1	No	2.4	612	Hypo	RA§/RA	46, XY	0.5	No	Yes (1501)
2	55/F	1	No	1.6	1308	Normo	RA§/RA	46, XX	0.5	Yes	Yes (1502)
3	61/F	1	Dependent	1.2	906	Normo	RA§/RA	46, XX	0.5	Yes	Yes (1502)
4	31/M	1	No	4.6	957	Hyper	RA§/RA	46, XY	0.5	Yes	Yes (1501)
5	52/F	1	No	1.5	1240	Hyper	RA§/RA	46, XX	0.5	Yes	Yes (1501)
6	31/M	14	No	2.2	1492	Hyper	RA§/RA	46, XY	0.5	No	No
7	40/F	12	Dependent	4.1	420	Hyper	RA/RA	46, XX	0.5	Yes	No
8	52/M	8	No	1.8	1717	Hyper	RA/RA	46, XY	0.5	No	No
9	47/F	5	No	6.4	1820	Hyper	RA/RCMD	46, XX	0.5	No	No
10	27/F	3	No	2.3	1930	Hyper	RA/RCMD	46, XX	0	Yes	No
11	44/M	168	Dependent	22.4	2155	Hyper	RA/RCMD	46, XY	0	NT	NT
12	55/F	1	Dependent	3.6	749	Hyper	RA/RCMD	46, XX	0.5	Yes	Yes (1501)
13	67/F	5	Dependent	1.2	687	Normo	RA/RCMD	46, XX	0.5	No	No
14	55/F	12	Dependent	5.1	1237	Hyper	RA/RCMD	46, XX	0.5	No	Yes (1501)
15	64/M	5	No	6.7	765	Hyper	RA/RCMD	47, XY, +Y, add(9)(q13)	1	No	Yes (1501)
16	50/M	12	Dependent	8.1	340	Hypo	RA/RCMD	47,XY +8	1	No	Yes (1501)
17	50/M	14	Dependent	4.5	70	Hyper	RAEB/RAEB-1	46, XY	1	No	No
18	35/M	2	Dependent	13.2	2153	Hyper	RAEB/RAEB-1	46, XY	0.5	No	No
19	52/M	1	Dependent	2.2	1358	Normo	RARS/RCMDRS	46, XY	0.5	No	Res (1502)
20	63/M	2	No	7.4	901	Hyper	RA/RCMD	46,XY, del20q	0.5	Yes	No

*UPN indicates unique patient number; PLT, platelet count ($\times 10^4/\mu L$); Neut, neutrophil count ($/\mu L$); BM, bone marrow; FAB, French-American-British classification; WHO, World Health Organization classification; IPSS, International prognostic scoring system for myelodysplastic syndromes; PNH, paroxysmal nocturnal hemoglobinuria; HLA-DR; D-related human leukocyte antigen; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; NT, not tested; RAEB, refractory anemia with excess of blasts; RARS, refractory anemia with ringed sideroblasts; RCMD-RS, RCMD with ringed sideroblasts.

†Interval between diagnosis and enrollment.

‡Average of 2 measurements at least 2 weeks apart.

§Patients showing bone marrow morphology as RA with minimal dysplasia.

Table 2.
Outcome of Treatment with Cyclosporine A (CSA)*

UPN	HI at 24th Week (Duration of CSA before Achieving HI) HI-E	HI-P	HI-N	Therapy after Study Period	Present Status
1	Major (8 wks)	Minor (6 wks)	No	Unknown	Lost follow-up at 6 mo
2	Minor† (6 wks)	Minor† (4 wks)	No	CSA	Alive at 30 mo, continuous response
3	Minor† (24 wks)	Minor† (24 wks)	Major (6 wks)	CSA	Alive at 36 mo, continuous response
4	Major (2 wks)	Major (4 wks)	No‡ (18 mo)	CSA	Alive at 36 mo, continuous response
5	No‡ (24 mo)	Minor† (20 wks)	No	CSA	Alive at 36 mo, continuous response
6	No	No	No	Androgen	Alive at 24 mo
7	No‡ (24 mo)	No‡ (10 mo)	No	CSA	Alive at 24 mo, continuous response
8	No	No	—	Observation	Alive at 36 mo
9	No	No	—	Observation	Alive at 36 mo
10	—	Minor (4 wks)	—	CSA	Alive at 36 mo
11	Minor† (22 wks)	—	—	CSA	Alive at 24 mo, continuous response
12	No	No	No	Observation	Alive at 30 mo
13	Major (16 wks)	No‡ (14 mo)	No‡ (14 mo)	CSA	Alive at 24 mo, continuous response
14	Major (18 wks)	No	No	CSA	Alive at 30 mo, continuous response
15	No	No	No	Observation	Alive at 36 mo
16	Major (8 wks)	No	No	mPSL, androgen	Alive at 30 mo, lost response
17	No	No	No	Observation	Died at 7 mo
18	No	—	—	Allo-SCT	WT-1 value elevated
19	No	No	No	Allo-SCT	Grade 4 cytopenia developed
20	NE	NE	NE	Androgen	Cyclosporine withheld due to infection, alive at 18 mo

*UPN indicates unique patient number; HI, hematologic improvement according to the response criteria from the International Working Group; HI-E, erythroid response; HI-P, platelet response, HI-N, neutrophil response; mPSL, methyl-predonisolone; allo-SCT, allogeneic stem cell transplantation.
†Maximal hematologic response turned out to be the most major until the latest follow-up.
‡Hematologic response was obtained until the latest follow-up.

as HI-E as well. Hematologic responses were durable and progressive. Figure 1 shows the kinetics of hematologic increments in patients who showed responses before the latest follow-up. Except for one patient (No. 10), a minor response at the 24th week turned out to be the most major response at the time of the latest follow-up. The patient (No. 10) has had a continued minor response for 36 months. In addition, many responders gained further responses that were not attained by patients treated with CSA for 24 weeks. Until now, 2 erythroid, one platelet, and 2 neutrophil responses have been further documented. In addition, one patient (No. 7), who was judged as a nonresponder at the 24th week, continued to take CSA thereafter, and obtained platelet and erythroid responses at the 10th and 24th months, respectively.

3.6. Analysis for the Presence of Minor T-Cell Clones

To gain insights into the mechanism of CSA-induced hematological responses, the presence or absence of minor T-cell clones was examined. We could examine the pretreatment T-cell repertoires in 13 patients, and samples drawn at the 24th week of CSA therapy were also available in 6 of them. Before CSA treatment, 11 patients showed skewed complementarity-determining region 3 spectratypes, and 2 patients displayed normal patterns. The presence or absence as well as the number of minor T-cell clones did not correlate with therapeutic outcomes (data not shown). As shown in Figure 2, the comparison of TCR-Vβ spectratypes between those obtained at pretreatment and after 24 weeks of CSA demonstrated that abnormally expanded minor T-cell clones present before CSA therapy persisted with a similar frequency even in CSA responders.

4. Discussion

In this prospective trial, 10 of 19 patients available for evaluation (53%) showed HI within 24 weeks of CSA therapy, and their responses lasted for at least 2 years if CSA was continuously administered. Reports documenting the efficacy of CSA against low-risk MDS are limited. In addition, most reports were single center experiences or a retrospective survey. Only 3 multicenter prospective trials exist, which reported

Table 3.
Adverse Events Observed within 6 Months of Cyclosporine A Treatment

	Grade According to CTC Version 2.0				
	0	1	2	3	4
Cardiovascular	19	1	0	0	0
Infectious	16	0	2	1*	1†
Renal	9	10	1	0	0
Hepatic	8	10	1	1*	0
Gastrointestinal	17	2	1	0	0
Metabolic	8	11	1	0	0
Cutaneous	17	3	0	0	0
Neutropenia	18	0	1	1†	0
Thrombocytopenia	18	1	0	1	0
Anemia	19	0	0	0	1

*†Adverse events developed in the same patients.

Table 4.
Pretreatment Variables Associated with Hematologic Response at 24th Week*

	Overall Response			Erythroid Response			Platelet Response		
	Responder	Nonresponder	*P*	Responder	Nonresponder	*P*	Responder	Nonresponder	*P*
Age, y			.81			.96			1
More than 50	5	4		4			3	5	
50 or younger	5	5		4	6		3	6	
Disease duration			.83			1			.009
4 mo or longer	4	5		4	5		0	8	
Less than 4 mo	6	4		4	5		6	3	
Karyotype			1			1			.51
Normal	9	8		7	9		6	9	
Abnormal	1	1		1	1		0	2	
PNH-type cells			.33			.64			.03
Yes	5	2		3	3		5	2	
No	4	7		4	7		1	9	
HLA DRB1 1501			.33			.35			.64
Yes	5	2		4	3		3	4	
No	4	7		3	7		3	7	
RA with minimal dysplasia			.44			.71			.03
Yes	5	2		4	3		5	2	
No	5	7		4	7		1	9	

*See Table 1 for abbreviations.

exceedingly high CSA response rates. Janasova demonstrated a response rate of 82% (14/17) [17], Dixit showed that 14 of 19 patients (74%) gained HI according to IWG criteria [19], and Chen reported the response rate at 62.5% (20/32) [23]. Although Atoyebi reported unsuccessful outcomes in 6 patients with FAB-RA and RARS [22], inconsistent results are inevitable in small-sized studies, as documented in studies using ATG [12,33]. The response rate of 53% in our study is consistent with previous reports as well as our retrospective survey [24]. Low-risk MDS, especially FAB-RA, is highly diverse in both clinical presentations and pathophysiology. In addition, a recent report from Matsuda demonstrated that the frequency of WHO-RA among FAB-RA is much higher in Japanese than in German patients [34,35]. Thus, comparison of response rates in patients of different ethnic background seems to have limited value. Rather, the predictive variables of the CSA response, which can be universally applicable, should be elucidated and compared between trials. We found that 3 variables: the presence of PNH-type cells, short duration of illness (less than 4 months), and the diagnosis of RA with minimal dysplasia, were significantly associated with the platelet response at the 24th week. In contrast, we could not find any predictive variables for overall and erythroid responses. The predictive value of having HLA-DRB1 1501, which was associated with the CSA response in our retrospective survey, was not confirmed in this study.

To our surprise, hematologic responses were durable and progressive after the 24th week of evaluation. Six additional HI were documented in four patients after the 24th week. RA patients with minimal dysplasia or PNH-type cells gathered into one cohort (RAminiD/PNH cohort) (UPN 1-7, 10, 12, 20) had an elevated probability of multi-lineage responses. HI was obtained in 7 of 9 patients, and 6 of 7 responses were multilineage. In particular, the platelet response was almost restricted in this cohort. Some patients in the other cohort, who showed neither the feature of RA with minimal dysplasia nor PNH-type cells (No. 8, 9, 11, 13-19), also gained a hematologic response to CSA (hematologic response rate of 4/10). However, most responses were restricted to the erythroid lineage. The significance of the presence of PNH-type cells in the prediction of the response

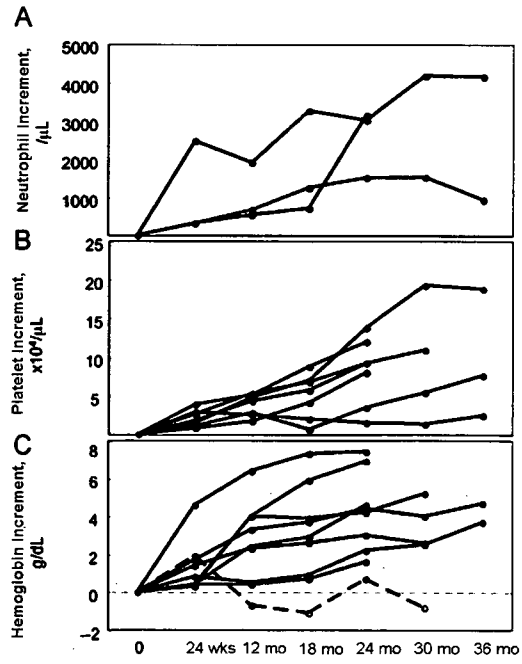

Figure 1. Hematologic increments in patients who showed a response up to the latest follow-up. A, Increments in neutrophil counts from baseline values in three patients who showed neutrophil responses. B, Increments in platelet counts in 7 responders. C, Increments in hemoglobin values in 9 responders. One patient who lost the response after the 12th month is indicated as a broken line.

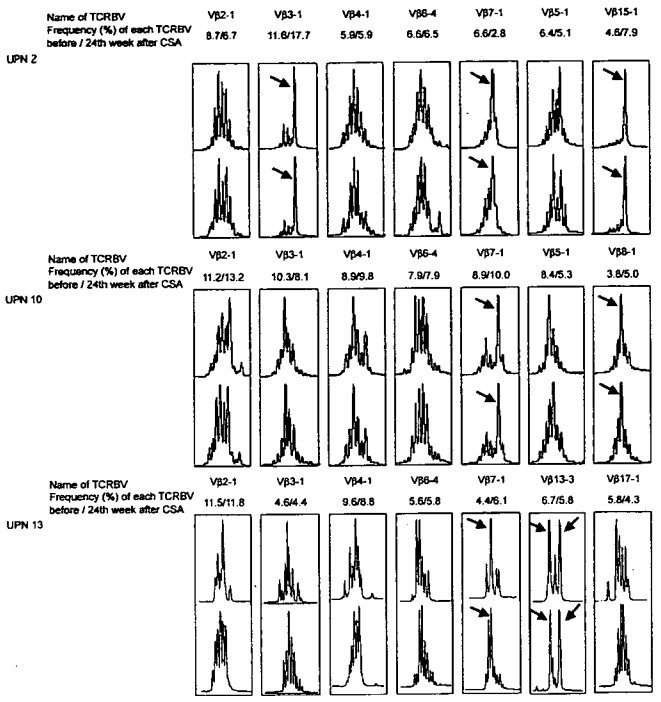

Figure 2. Representative T-cell receptor β chain variable region (TCR-Vβ) spectratype before and after cyclosporine A (CSA) therapy. The spectratypes listed were the eight frequent Vβ families; the frequency was defined by microplate hybridization assay. The name of TCR Vβ, the relative frequency of each Vβ, and spectratypes are shown. All patients shown here were CSA responders.

to immunosuppressive therapy against aplastic anemia as well as MDS has been reported [15,25,26]. A retrospective survey demonstrated that RA patients harboring PNH-type cells showed less pronounced morphologic abnormality, rare progression into acute leukemia, a higher incidence of HLA-DR15, and higher CSA response rate [26]. The results of this study are in agreement with previous observations.

Molldrem and Kochenderfer showed that MDS patients exhibited a skewed TCR-Vβ repertoire, indicating the presence of a clonal T-cell population. The clonal population was diminished only in responders to immunosuppressive therapy and ATG [36,37], which is consistent with clinical findings that ATG produces long-lasting HI without additional therapy. In this study, we also detected the presence of abnormally expanded T-cell clones in most MDS patients. However, CSA administration for 24 weeks did not affect expanded T-cell clone frequencies, even in responders. Thus, CSA does not appear to eliminate pathologic T-cells, but inhibits their marrow-suppressive function, thus bringing about a CSA-dependent hematological response, as reported in aplastic anemia [38].

In addition to its promising effects, CSA has a limited toxicity profile and can be safely administered in outpatient clinics. CSA produced less severe adverse events than ATG or lenalidomide, which caused effects defined as over grade 2 toxicities. Stadler reported that the administration of horse- or rabbit-derived ATG produced higher than grade 2 toxicities in 23 of 35 MDS patients [13]. Higher than grade 2 neutropenia (65%) or thrombocytopenia (53%) was documented in a phase I trial of lenalidomide [9]. Most of the adverse events seen in CSA-treated patients were slightly elevated liver enzymes or marginally impaired renal function tests (elevated creatinine or potassium values); dose reduction or interruption of CSA was rarely needed.

In conclusion, the use of CSA was associated with HI in selected patients with FAB-RA without severe adverse events. Patients harboring minor populations of PNH-type cells or showing minimal dysplastic features have an elevated likelihood of recovering from thrombocytopenia and anemia. Erythropoiesis is generally restored in patients who do not have PNH-type cells. As the hematologic response is CSA-dependent, long-term outcomes, including the possibility of accelerating leukemic transformation, must be carefully observed.

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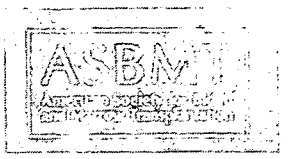

Growth and Differentiation Advantages of CD4$^+$OX40$^+$ T Cells from Allogeneic Hematopoietic Stem Cell Transplantation Recipients

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ABSTRACT

OX40 (CD134), an activation-induced costimulatory molecule, is mainly expressed on CD4$^+$ T cells. Several reports, including previous reports from our laboratory, suggest that OX40-mediated signaling plays an important role in the development of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (Allo HSCT). Here, we show that peripheral blood CD4$^+$OX40$^+$ T cells are a unique cell subset as they possess the homing receptors of lymph nodes, and some of them have an exceptional capacity to produce high levels of interleukin-2 (IL-2) upon the stimulation through T cell receptors. Stimulation with IL-7 acts selectively on CD4$^+$OX40$^+$ T cells not only to induce antigen-independent growth but also to increase the frequency of cells with IL-2-producing potential. Simultaneous, but not sequential, ligation of the T cell receptor and OX40 induces CD4$^+$OX40$^+$ T cells to produce far more IL-2, which causes them to proliferate abundantly and differentiate readily into Th1- or Th2-biased effector memory T cells, especially in Allo HSCT recipients. Although not all the CD4$^+$OX40$^+$ T cells had IL-2-producing capacity, Allo HSCT recipients with chronic GVHD (cGVHD) had a significantly higher frequency of IL-2-producing OX40$^+$ cells in their peripheral blood CD4$^+$ T cell subset than Allo HSCT recipients without cGVHD. Collectively, CD4$^+$OX40$^+$ T cells with IL-2-producing potential are expected to be privileged for growth and differentiation in lymph nodes upon antigen presentation, suggesting that they might be involved in the process of inducing or maintaining cGVHD.

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KEY WORDS

OX40 CD4 T cell Allogeneic hematopoietic stem cell transplantation Chronic graft-versus-host disease (GVHD)

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) remains a serious complication that affects long-term survivors of allogeneic hematopoietic stem cell transplantation (Allo HSCT). It is not only the leading cause of nonrelapse mortality (NRM), it is also associated with decreased quality of life [1]. To prevent and treat GVHD, immunosuppressive agents such as calcineurin inhibitors are generally used, which increases the risk of developing opportunistic infections. It has been shown that GVHD is initiated by donor-derived CD4$^+$ and CD8$^+$ T cells that recognize a subset of host antigens [2,3]. Indeed, it has been shown that ex vivo depletion of the T cells in the graft effectively reduces the incidence and severity of acute GVHD (aGVHD) [4,5]. Unfortunately, this technique is also associated with increased incidences of graft rejection, relapses, and infectious complications, which prevents it from being widely used. Another technique to specifically deplete donor-derived alloreactive T cells that is currently being developed involves stimulating the graft with recipient cells in vitro and then depleting the activated T cells with monoclonal antibodies (mAb) [6-8]. However, such depletion-based techniques would probably fail to prevent cGVHD because the alloreactive T cells that cause cGVHD are believed to be derived from hematopoietic stem cells (HSC) in the graft rather than already being mature T cells [9-12]. A better way to prevent and treat

cGVHD would be to first identify which alloreactive cells are directly responsible for this disease; these cells could then be readily detected in the blood and specifically depleted within the host.

OX40 (CD134) is a member of the tumor necrosis factor (TNF) receptor superfamily [13], and is an activation-induced antigen that is predominantly expressed on CD4$^+$ T cells [14]. The ligand for OX40 (OX40L) is mainly expressed on activated antigen-presenting cells (APCs) such as dendritic cells and B cells [15-17]. OX40 signaling acts as an important costimulatory signal, as it augments interleukin 2 (IL-2) production [18,19], prolongs cell survival by upregulating Bcl-2 and Bcl-x_L expression [20], induces the clonal expansion of naïve CD4$^+$ T cells [19,21], and generates memory T cells by promoting the survival of effector T cells [19,22,23]. OX40-mediated signaling is also indispensable for expanding memory T cells in secondary immune responses and prolonging their survival [24]. A large body of evidence suggests that OX40-mediated signaling plays a pivotal role in the development of several immune-mediated conditions such as experimental autoimmune encephalomyelitis [16,25], collagen-induced arthritis [26], allergic lung inflammation [24,27], inflammatory bowel disease [28], and GVHD [29,30]. Because the in vivo blockade of OX40-mediated signals ameliorates these diseases in murine models, it is possible that targeting OX40 may also be useful for treating human diseases [14].

Buenafe et al [31] reported that the antigen-specific T cells in the spinal cord of Lewis rats displaying experimental autoimmune encephalomyelitis are frequently CD4$^+$OX40$^+$ T cells. Tittle et al [32] showed that CD4$^+$OX40$^+$ T cells are the alloreactive T cells in a murine GVHD model. In addition, we previously showed that the occurrence of cGVHD correlates positively with the frequency of peripheral blood CD4$^+$OX40$^+$ T cells [33]. Consequently, we speculated that the circulating CD4$^+$OX40$^+$ T cell subset of Allo HSCT recipients contains alloreactive T cells that are involved in the process of inducing and maintaining cGVHD. To further understand the role CD4$^+$OX40$^+$ T cells play in the development of cGVHD, we here isolated the CD4$^+$OX40$^+$ T cells from Allo HSCT recipients and healthy volunteers (HVs) and assessed their characteristics.

SUBJECTS, MATERIALS, AND METHODS

Subjects

Peripheral blood samples were obtained from 13 HVs and 43 Allo HSCT recipients who had undergone transplantation at least 100 days previously. Each subject gave written informed consent. Allo HSCT recipients were required to be in complete donor chimerism as well as in complete remission at the time of sampling. The clinical characteristics of the Allo HSCT recipients are summarized in Table 1. Standard conditioning for patients with hematologic malignancies consisted of 12 Gy total-body irradiation (TBI) and cyclophosphamide (Cy; 120 mg/kg), 12 Gy TBI and melphalan (Mel; 140 mg/m^2), or busulfan (Bu/Cy; 16 mg/kg) and Cy (120 mg/kg). Patients with aplastic anemia (AA) received 200 mg/kg Cy and antithymocyte-globulin (ATG), and a patient with adrenoleukodystrophy was treated with Bu (8 mg/kg), Cy (120 mg/kg), and 7.5Gy total lymphoid irradiation [34]. Reduced-intensity conditioning (RIC) was performed using 2-4 Gy TBI, fludarabine (Flu; 125 mg/m^2), and either Bu (8 mg/kg) or Mel (80-140 mg/m^2). The presence of cGVHD in Allo HSCT recipients was defined as the presence of active symptoms, for which immunosuppressive therapy was required [1,35]. In other words, patients defined as positive for cGVHD included patients with extensive cGVHD and patients with limited cGVHD, which manifests itself as significant hepatic dysfunction (value of Alkaline Phosphatase greater than twice the normal upper limit). All studies involving these blood samples were approved by the institutional review board of Kyoto University.

Table 1. *Patient Characteristics*

Characteristics	Data
No. male/no. female	20/23
Median age, years (range)	52 (25-74)
Diagnosis, no. (%)	
Acute lymphoblastic leukemia	2 (5)
Acute myelogenous leukemia	12 (28)
Myelodysplastic syndrome	7 (16)
CML/MPD	7 (16)
Adult T cell Leukemia	1 (2)
Lymphoma	8 (19)
Myeloma	3 (7)
Aplastic anemia	2 (5)
Adreno-leukodystrophy	1 (2)
Donor type, no. (%)	
Matched related	19 (44)
Matched unrelated	16 (37)
Mismatched related	5 (12)
Mismatched unrelated	3 (7)
Conditioning regimen, no. (%)	
Standard	24 (56)
Reduced intensity	20 (44)
Stem cell source, no. (%)	
Bone marrow	27 (62)
Peripheral blood	14 (33)
Cord blood	2 (5)
cGVHD, no. (%)	
Yes	23 (53)
No	20 (47)
Immunosuppression at the time of analysis, no. (%)	29 (67)
Median time after Allo HSCT for analysis, mo (range)	12 (4-149)

CML/MPD indicates chronic myelogenous leukemia/myeloproliferative disorder; cGVHD, chronic graft-versus-host disease; Allo HSCT, allogeneic hematopoietic stem cell transplantation.

mAb and Flow Cytometric Analysis

An anti-OX40 mAb (131, mouse IgG1) was established in our laboratory [36]. It was used either as a purified protein or it was biotinylated by using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). For flow cytometric analysis, cells were incubated with appropriate concentrations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated mAbs in the dark at 4 C for 20 minutes. The cells were then washed twice and analyzed by flow cytometry on FACSCalibur (BD Biosciences, San Jose, CA) with CELLQuest software (BD Biosciences). PE-conjugated anti-OX40 (PE-anti-OX40), APC-anti-CD45RA, FITC- and PE-anti-interferon γ (IFN-γ), PE-anti-interleukin 4 (IL-4), and Alexa 647-anti-IL7Rα (CD127) were purchased from BD Pharmingen (San Diego, CA). FITC-anti-CD45RO, FITC-anti-CD25, PE-anti-IL-2, FITC-anti-IL-4, and all the isotype-matched control mAbs were obtained from eBioscience (San Diego, CA). FITC-anti-CD62L and FITC-anti-CCR7 were obtained from Beckman Coulter (Fullerton, CA) and R&D systems (Minneapolis, MN), respectively. Intracellular Foxp3 staining was performed by using the PE-conjugated anti-human Foxp3 staining set (PCH101, eBioscience) according to the manufacturer's instructions. Staining of cytoplasmic phosphorylated STAT5 was performed by using Alexa 488-anti-phospho-STAT5 (clone 47, BD Biosciences) according to the manufacturer's instructions. To detect apoptotic cells and dead cells, the cells were stained with 1 μg/mL propidium iodide (PI, Sigma-Aldrich, St. Louis, MO) for 15 minutes at room temperature. For cell proliferation analysis, the cells were labeled for 10 minutes at room temperature with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) at a final concentration of 5 μM in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich).

T Cell Isolation and Sorting

Peripheral blood mononuclear cells (PBMC) were isolated from HVs and Allo HSCT recipients by using Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. The $CD4^+$ T cells were isolated with CD4 Multisort Kit (Miltenyi Biotec Bergisch Gladbach, Germany) and their purity exceeded 97%. $CD4^+$ T cells were stained with biotinylated anti-OX40 mAb followed by APC-streptavidin (eBioscience) and sorted into $OX40^+$ and $OX40^-$ fractions by FACSAria (BD Biosciences) with FACSDiVA 4.1 software (BD Biosciences). In some experiments, the cells were also stained with PE-anti-CD45RA (BD Pharmingen) and sorted into $OX40^+$ memory ($CD45RA^-OX40^+$) and $OX40^-$ memory ($CD45RA^-OX40^-$) cells. The sorted T cell subsets were more than 90% pure.

Flow Cytometric Analysis of Intracellular Cytokines

For intracellular cytokine staining, cells were suspended in culture medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 10% fetal calf serum (FCS, Hyclone, Logan, UT) and 1% penicillin-streptomycin-glutamine mixture (Invitrogen) and stimulated for 6 or 16 hours in plates coated with anti-CD3 mAb (OKT3, 10 μg/mL) with or without anti-OX40 mAb (131, 10 μg/mL) in the presence of 2 μg/mL soluble anti-CD28 mAb (H046, mouse IgG1, agonistic antibody established in our laboratory; T. Hori, unpublished data) (αCD3/28 or αCD3/28/OX40 stimulation). Brefeldin A (BFA, Sigma-Aldrich) was added at a concentration of 10 μg/mL for the last 4 hours. In some experiments, cells were stimulated with PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) for 4 hours in the presence of BFA (PMA/Iono stimulation). After stimulation, the cells were washed twice, surface stained with the appropriate mAbs, and fixed with 2% formaldehyde (Wako Pure Chemical Industries, Osaka, Japan) diluted in PBS. The cells were then permeabilized with 0.2% saponin (Sigma-Aldrich)-containing buffer and intracellular cytokine levels were measured by using the relevant mAbs.

Assessment of Cytokine Release by ELISA

To detect IL-2 in the culture supernatants, cells at a concentration of 5 10^5/mL were stimulated for 24 hours with αCD3/28 or αCD3/28/OX40 as described above. The IL-2 levels in the medium were measured by enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-human IL-2 polyclonal Ab (Pierce Biotechnology, Rockford, IL), a biotinylated-anti-human IL-2 mAb (BG5, mouse IgG1, Pierce Biotechnology), and a recombinant human IL-2 standard (Pierce Biotechnology) according to the manufacturer's instructions.

T Cell Culture and Stimulation

For experiments using interleukin 7 (IL-7), cells were suspended in culture medium in the presence or absence of 1 ng/mL IL-7 (Peprotech, Rocky Hill, NJ) for 5 days. For polyclonal stimulation and expansion, isolated $CD4^+$ T cell subsets were suspended in culture medium at a concentration of 5 10^5/mL and stimulated for 12 hours with αCD3/28 or αCD3/28/OX40 in 48- or 96-well plates. The cells were then harvested, washed, and cultured for 4 days in culture medium. In some experiments, after 12 hours of stimulation followed by washing, the cells were cultured in plates coated with anti-OX40

mAb or control mouse IgG1 mAb (eBioscience) for 4 days.

Statistical Analysis

Results are expressed as means ± standard deviation (SD). The statistical significance of differences was determined by using a 2-sided paired *t*-test or Student's *t*-test. Differences with $P < .05$ were considered to be significant.

RESULTS

The CD4$^+$OX40$^+$ T Cell Subset Shares the Characteristics of Central Memory T Cells

We first tested the frequency of OX40-expressing peripheral blood CD4$^+$ T cells in Allo HSCT recipients and HVs by multicolor flowcytometry. Although OX40 is an activation-induced antigen, we found it was expressed on a considerable number of peripheral blood CD4$^+$ T cells from both groups. However, Allo HSCT recipients showed higher frequencies of OX40$^+$ cells (Figure 1A). Further analysis revealed that nearly all of the CD4$^+$OX40$^+$ T cells belonged to the CD45RO$^+$ memory subset and most also expressed CCR7 and CD62L (Figure 1B and C), which indicates that they are central memory T cells. There were no significant differences in the CD4$^+$OX40$^+$ T cells from HVs and Allo HSCT recipients in terms of their CD45RO, CCR7, and CD62L expression. As CD4$^+$CD25$^+$ regulatory T cells are also reported to express OX40 [37-41], we determined the intracellular Foxp3 levels in the CD4$^+$CD25$^+$ T cells and CD4$^+$OX40$^+$ T cells. Although a considerable proportion of the CD4$^+$CD25$^+$ T cells were Foxp3$^+$, fewer than 10% of the CD4$^+$OX40$^+$ T cells from both Allo HSCT recipients and HVs were Foxp3$^+$ (Figure 1D). Recent reports indicated that regulatory T cells have reduced expression of CD127 (IL-7R alpha chain, IL-7Rα) [42]. We also found that the majority of CD4$^+$CD25^{high} T cells were IL-7Rα^{low}. However, CD4$^+$OX40$^+$ T cells were almost all IL-7Rα^{high} (Figure 1D). Collectively, CD4$^+$OX40$^+$ T cells include a minor population of regulatory T cells.

To further characterize the CD4$^+$OX40$^+$ T cells, CD4$^+$ T cells that were freshly isolated from HVs and Allo HSCT recipients were sorted into OX40$^+$ and OX40$^-$ fractions (Figure 2A), stimulated with αCD3/28 for 6 hours, and then subjected to intracellular cytokine staining (Figure 2B and C). There were no differences in the frequency of IL-2-producing cells in the CD4$^+$ T cell population upon αCD3/28 stimulation when CD4$^+$ T cells were isolated with magnetically labeled anti-CD4 mAb or negatively selected to enrich for CD4$^+$ T cells (data not shown). In addition, we confirmed that the binding of anti-OX40 mAb, clone 131, to the cell surface was not enough to affect the IL-2-producing capacity of the cells, because the addition of soluble anti-OX40 mAb to CD4$^+$ T cells did not alter the frequency of IL-2-producing cells after subsequent αCD3/28 stimulation (data not shown). There were marked differences between Allo HSCT recipients and HVs in terms of the cytokine profiles of their OX40$^-$ cells. Although there were very few IL-2-, IFN-γ-, or IL-4-producing cells in the OX40$^-$ cells from HVs, a large proportion (over 30%) of the OX40$^-$ cells from Allo HSCT recipients produced IFN-γ; a small population of these IFN-γ-producing cells (about 5%-10%) also produced IL-2. In contrast, about 10% of the OX40$^+$ cells from both the Allo HSCT recipients and HVs produced IL-2, whereas very few cells produced IFN-γ or IL-4. As we could not detect cells that produce IFN-γ and IL-4 simultaneously (data not shown), we defined the cells that produce IL-2 but not IFN-γ or IL-4 as $T_{IL\text{-}2}$ cells. The frequency of $T_{IL\text{-}2}$ cells was calculated as follows: (the frequency of all IL-2-producing cells) – (the frequency of IFN-γ- and IL-2-producing cells) – (the frequency of IL-4- and IL-2-producing cells). As shown in Figure 2C, $T_{IL\text{-}2}$ cells were only detected in the OX40$^+$ fraction of both Allo HSCT recipients and HVs.

Signaling from IL-7R and Crosslinking of OX40 Robustly Augments IL-2 Production by OX40$^+$ Memory T Cells

As OX40$^+$ cells exclusively exist within the memory cell fraction, we next compared the characteristics of OX40$^+$ memory cells and OX40$^-$ memory cells. To this end, CD4$^+$ T cells of HVs were stained with PE-anti-CD45RA and biotinylated-anti-OX40 Ab before adding streptavidin-APC and sorting them into CD45RA$^-$OX40$^+$ T cells and CD45RA$^-$OX40$^-$ T cells. We regarded the former as OX40$^+$ memory cells and the latter as OX40$^-$ memory cells.

IL-7 is known to be critically involved in maintaining memory CD4$^+$ T cell homeostasis through its ability to induce antigen-independent proliferation in the periphery [43,44]. We found that the addition of IL-7 to culture medium sustained and augmented OX40 expression on CD4$^+$ T cells (data not shown). We then investigated the association between surface expression of OX40 and its ability to produce IL-2. First, freshly sorted OX40$^+$ memory cells were cultured in growth medium unsupplemented with cytokines. Five days later, the cells had lost OX40 expression as well as IL-2-producing capacity in response to αCD3/28 stimulation (the left row of Figure 3A). After sorting and CFSE-labeling, OX40$^+$ memory cells from HVs were then cultured for 5 days in the presence of IL-7. The cells showed enhanced expression of OX40 and an increased frequency of IL-2-producing cells. Meanwhile, culture of OX40$^-$ memory cells with IL-7 neither induced the expression of OX40 nor enhanced the capacity of

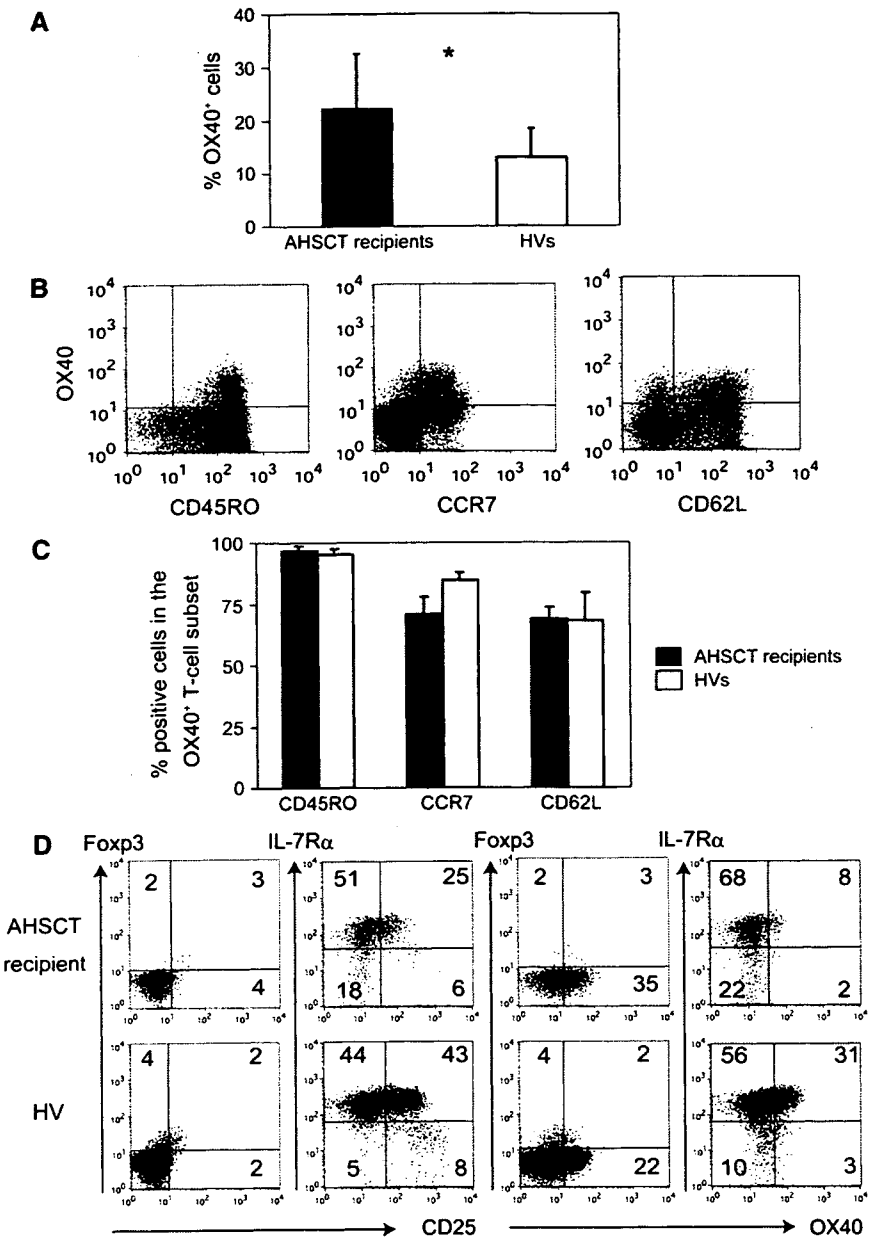

Figure 1. The $CD4^+OX40^+$ T cell subset contains central memory T cells. (A-C) Peripheral blood $CD4^+$ T cells of Allo HSCT recipients and HVs were analyzed for OX40, CD45RO, CCR7, and CD62L expression. (A) The frequencies of OX40-positive cells in the $CD4^+$ T cell subset from 36 Allo HSCT recipients and 13 HVs are shown as means ± SD. $^*P < .01$ (B) The dot plots shown are representative of 25 Allo HSCT recipients. (C) The frequencies of CD45RO-, CCR7-, and CD62L-positive cells in the $OX40^+$ T cell subset from 4 Allo HSCT-recipients and 3 HVs are shown as means ± SD. (D) $CD4^+$ T cells from Allo HSCT recipients and HVs were analyzed for correlations in their expression of CD25, OX40, intracellular Foxp3, and IL-7Rα. The dot plots shown are representative of 6 Allo HSCT recipients (upper) and 3 HVs (lower).

these cells to produce IL-2 (the right row of Figure 3A). Taken together, there seems to be a close association between the expression of OX40 and IL-2-producing capacity. In addition, a significant proportion of $OX40^+$ memory cells treated with IL-7 showed a decreased CFSE staining intensity, indicating that they had begun to proliferate. To determine whether IL-7 differentially delivered signals downstream of IL-7R, the phosphorylation status of cytoplasmic STAT5 was analyzed. As shown in Figure 3B, STAT5 was phosphorylated equally well in $OX40^+$ memory cells and $OX40^-$ memory cells upon IL-7 stimulation.

As previously reported [18], crosslinking of OX40 in addition to αCD3/28 stimulation resulted in a remarkable increase in the amount of IL-2 production by $OX40^+$ memory cells (Figure 4A). When we examined the IL-2 production of $OX40^+$ memory cells at 2-6 and 12-16 hours after stimulation, the costimulation through OX40 increased the frequency of IL-2-producing cells over time from 7% at 2-6 hours to about 13% at 12-16 hours (Figure 4B).

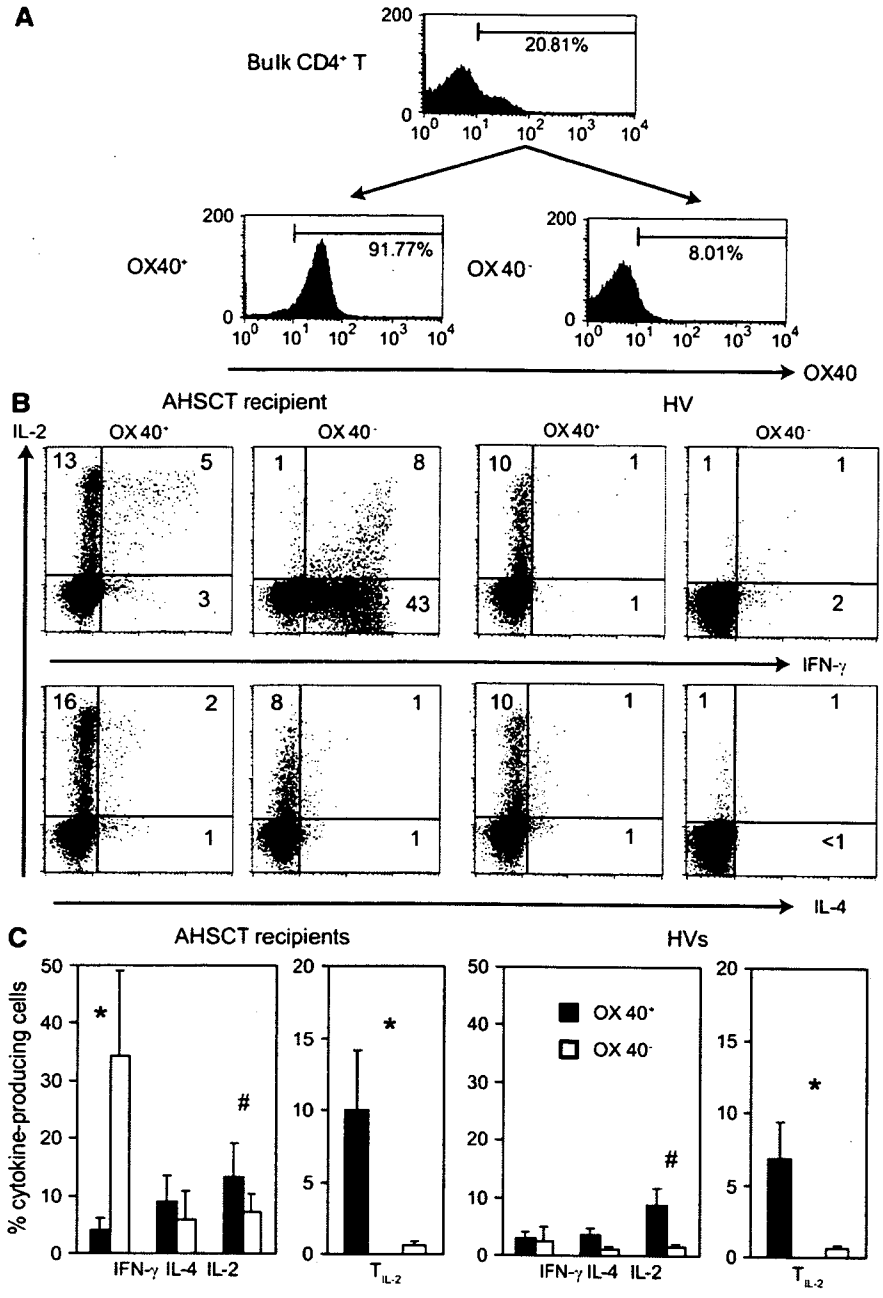

Figure 2. $CD4^+OX40^+$ T cells show an increased propensity to produce IL-2 in response to antigenic stimulation. (A) $CD4^+$ T cells from Allo HSCT recipients and HVs were sorted into $OX40^+$ and $OX40^-$ fractions. (B) Cytokine production by $OX40^+$ and $OX40^-$ cells stimulated with αCD3/28. The data shown are representative of 10 Allo HSCT recipients (left) and 9 HVs (right). (C) The frequencies of cytokine-producing $OX40^+$ and $OX40^-$ cells are shown as means ± SD. T_{IL-2} cells were defined as the cells that produce IL-2 but not IFN-γ or IL-4. $*P < .01$, $\#P < .05$.

OX40-Mediated Signaling Enhances the Survival and Proliferation of $OX40^+$ Memory T Cells

Having demonstrated that $OX40^+$ memory cells produce massive amounts of IL-2 when the OX40-mediated signal is present during antigenic stimulation, we next examined the effects of OX40-mediated signaling on the survival and proliferation of $OX40^+$ memory cells. For this, sorted $OX40^+$ memory cells from Allo HSCT recipients were labeled with CFSE, stimulated with αCD3/28 or αCD3/28/OX40 for 12 hours, washed, and then cultured for 4 days in growth medium without exogenous cytokines. As a control, $OX40^-$ memory cells were treated similarly. The intensity of the CFSE signal was analyzed to determine the degree to which the cells had proliferated, while their positivity for propidium iodide was analyzed to determine their susceptibility to apoptosis. The $OX40^-$ memory cells of Allo HSCT recipients did not proliferate and lost their viability during the course of cell cultivation (the left row of Figure 5A and B).

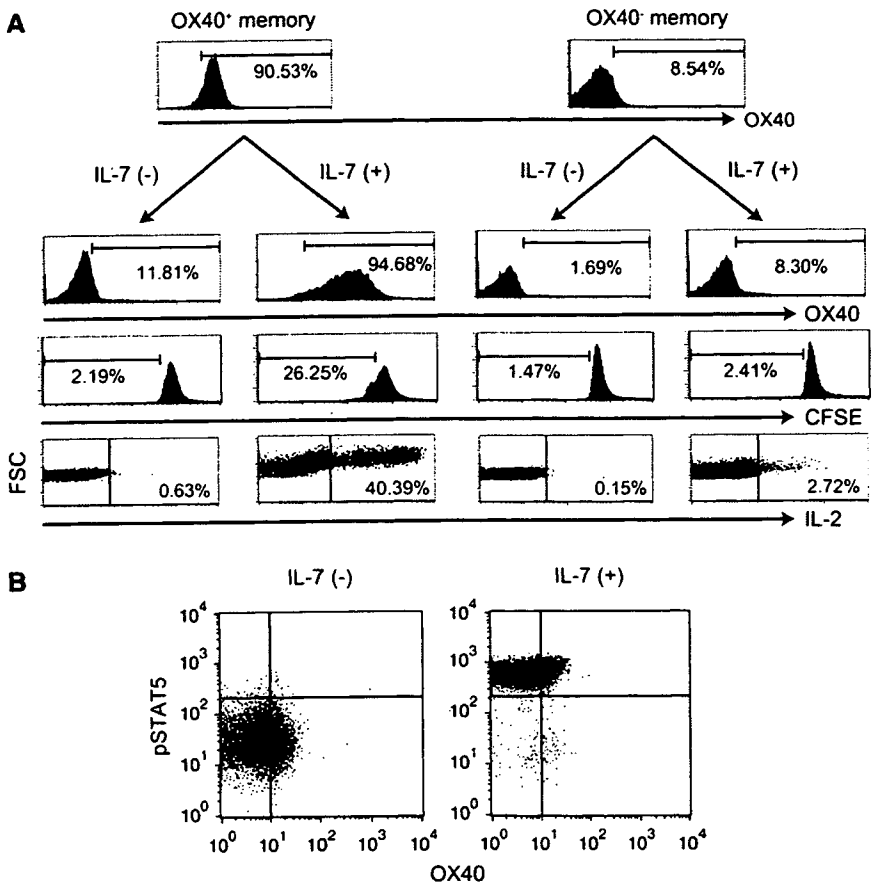

Figure 3. OX40$^+$ memory T cells have greater survival and proliferation potentials. (A) CD4$^+$ T cells from HVs were sorted into CD45RA$^-$OX40$^+$ T cells (OX40$^+$ memory T cells) and CD45RA$^-$OX40$^-$ T cells (OX40$^-$ memory T cells). Then, the cells were labeled with CFSE and cultured in the absence or presence of 1 ng/mL IL-7 for 5 days before measuring OX40 expression, division profile, and IL-2-production upon αCD3/28 stimulation. The dot plots shown are representative of 3 independent experiments. (B) CD4$^+$ T cells of HVs were incubated for 15 minutes in the absence or presence of 1 ng/mL IL-7 and analyzed for OX40 and cytoplasmic phosphorylated-STAT5 (pSTAT5). Data are representative of 3 HVs.

In contrast, OX40$^+$ memory cells showed substantial cell growth and high viability in response to αCD3/28 stimulation (the middle row of Figure 5A and B). When OX40 ligation was present during αCD3/28 stimulation, the OX40$^+$ memory cells showed explosive proliferation without impairment of cell viability (the right row of Figure 5A and B).

OX40 is transiently expressed upon TCR triggering, peaking at 48 hours and disappearing after 72 to 96 hours in vitro [19]. We also found that 12-hour stimulation with αCD3/28 induces the expression of OX40 on OX40$^-$ memory cells with similar kinetics. We next evaluated the effect of delivering the OX40-mediated signal after αCD3/28 stimulation (Figure 5C). For this, OX40$^+$ memory and OX40$^-$ memory cells from HVs were labeled with CFSE, and then stimulated with αCD3/28 or αCD3/28/OX40 for 12 hours. After washing, the cells were cultured in plates coated with mouse IgG1 (Figure 5C, upper dot plots and histograms) or anti-OX40 Ab (Figure 5C, lower dot plots and histograms) for an additional 4 days. When the OX40$^+$ memory cells were stimulated with αCD3/28 before incubation with anti-OX40 Ab, a marginal increase in the proportion of CFSE-low and PI-negative cells was seen (the middle row of Figure 5C). As for OX40$^-$ memory cells, despite the acquisition of OX40, they neither showed enhanced proliferation nor increased cell viability when the OX40-mediated signal was subsequently added (the left row of Figure 5C). Thus, for CD4$^+$ T cells to maintain their viability and expand efficiently, antigenic and OX40-mediated signals must be present simultaneously.

Effect of OX40-Mediated Signaling on Differentiation into Effector Memory T Cells

As CD4$^+$OX40$^+$ T cells are central memory T cells, we next investigated whether OX40-mediated signaling promotes their differentiation into effector memory T cells. For this, OX40$^-$ memory and OX40$^+$ memory cells were stimulated for 12 hours with αCD3/28 or αCD3/28/OX40, washed, cultured in medium without exogenous cytokines for 4 days, and then analyzed for their cytokine profiles. As shown

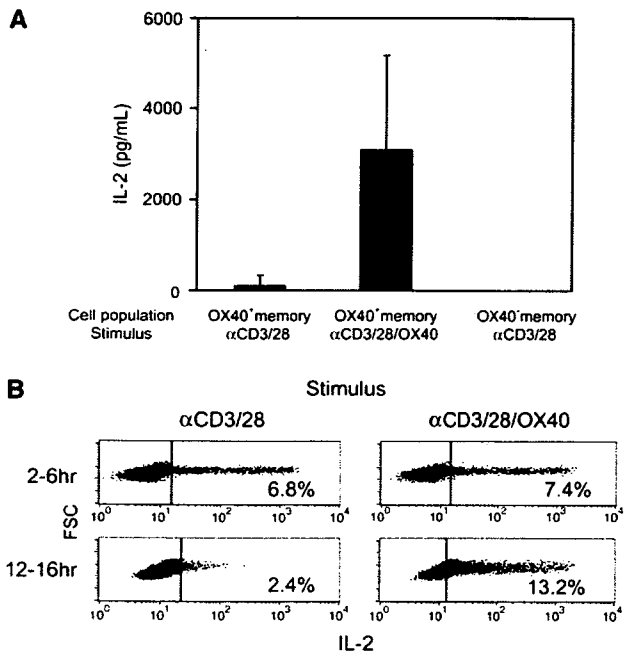

Figure 4. Crosslinking of OX40 enables $OX40^+$ memory T cells to produce massive amounts of IL-2 for an extended time period. (A,B) $CD4^+$ T cells from HVs were sorted into $CD45RA^-OX40^+$ T cells ($OX40^+$ memory T cells) and $CD45RA^-OX40^-$ T cells ($OX40^-$ memory T cells). (A) The cells were subjected to αCD3/28 or αCD3/28/OX40 stimulation for 24 hours. The levels of IL-2 secreted into the culture supernatants were measured by ELISA and are shown as means ± SD. (B) $OX40^+$ memory T cells of HVs were stimulated with αCD3/28 or αCD3/28/OX40 and their IL-2-production 2-6 hours (upper) and 12-16 hours (lower) later was analyzed. The data shown are representative of 3 independent experiments.

in the right row of Figure 6A, the majority of $OX40^+$ memory cells from HVs given αCD3/28/OX40 stimulation did not produce IFN-γ or IL-4. In contrast, the αCD3/28/OX40-stimulated $OX40^+$ memory cells from Allo HSCT recipients showed substantial differentiation into effector memory T cells (the right row of Figure 6B). Interestingly, the directions of polarization differed between the patients: $OX40^+$ memory cells from Case 1, a patient with refractory multiorgan cGVHD, mainly differentiated into the Th1 direction, whereas those from Case 2, a patient with pulmonary cGVHD, differentiated mainly into Th2-type cells. These results suggest that OX40-mediated signaling induces $OX40^+$ memory T cells from Allo HSCT recipients not only to proliferate, but also to differentiate into effector memory T cells.

Allo HSCT Recipients with cGVHD Have Much Higher Frequencies of IL-2-Producing $OX40^+$ Cells among $CD4^+$ T Cells Than Allo HSCT Recipients without cGVHD

As for the detection of intracellular cytokines, PMA/Iono stimulation has been widely used [45]. Compared with αCD3/28 stimulation, we could detect cytokine-producing cells more frequently when $CD4^+$ T cells from Allo HSCT recipients and HVs were stimulated with PMA/Iono. As shown in Figure 7A, PMA/Iono stimulation of $CD4^+$ T cells resulted in massive IL-2 production not only in $OX40^+$ cells but also in $OX40^-$ cells, which was not observed upon αCD3/28 stimulation. This suggests that PMA/Iono stimulation could promote IL-2-production even in cells that are not prepared for TCR-mediated signaling. Notably, we found that the $CD4^+OX40^+$ T cells were heterogeneous in their ability to produce IL-2 in response to PMA/Iono stimulation. We then examined the frequency of $OX40^+$ cells capable of producing IL-2 in Allo HSCT recipients with (n = 15) or without (n = 10) cGVHD. As BFA was continuously present during the stimulation, there was no increase in cell surface OX40 molecules during PMA/Iono stimulation. Consequently, we not only examined how OX40 expression on its own relates to cGVHD (left panel of Figure 7B), we also examined the correlation between the frequency of $IL\text{-}2^+OX40^+$ cells and the occurrence of cGVHD (right panel of Figure 7B). Higher frequencies of $OX40^+$ cells were observed on average in the cGVHD patients, which is consistent with our previous observations ($P = .032$) [33]. However, a closer correlation with cGVHD was detected when we examined the frequency of IL-2-producing $OX40^+$ cells ($P = .007$).

DISCUSSION

Since the concept of central memory and effector memory T cells was proposed [46], the heterogeneity of memory T cells has been an active area of research. Although the origin of central memory and effector memory T cells remains relatively poorly understood [47], it is generally accepted that central memory T cells produce IL-2, show high proliferative potential, and differentiate into cytokine-producing effector cells upon TCR triggering [48]. In this study, we found that circulating $CD4^+OX40^+$ T cells show these characteristics of central memory T cells, and they contain the cells that produce a large amount of IL-2 in response to αCD3/28 stimulation. Not only central memory T cells but also naïve and effector memory T cells have been shown to express OX40 in vivo [49,50]. However, we could not detect OX40 on any circulating naïve and effector memory $CD4^+$ T cells from Allo HSCT recipients or HVs. Although it is unclear why there is preferential expression of OX40 on central memory T cells in the circulating $CD4^+$ T cell population, we speculate as follows: naïve and central memory $CD4^+$ T cells that received activating signals in the lymphoid organs become effector cells or $OX40^+$ "activated" central memory T cells depending on the inflammatory status of the lymphoid tissues.

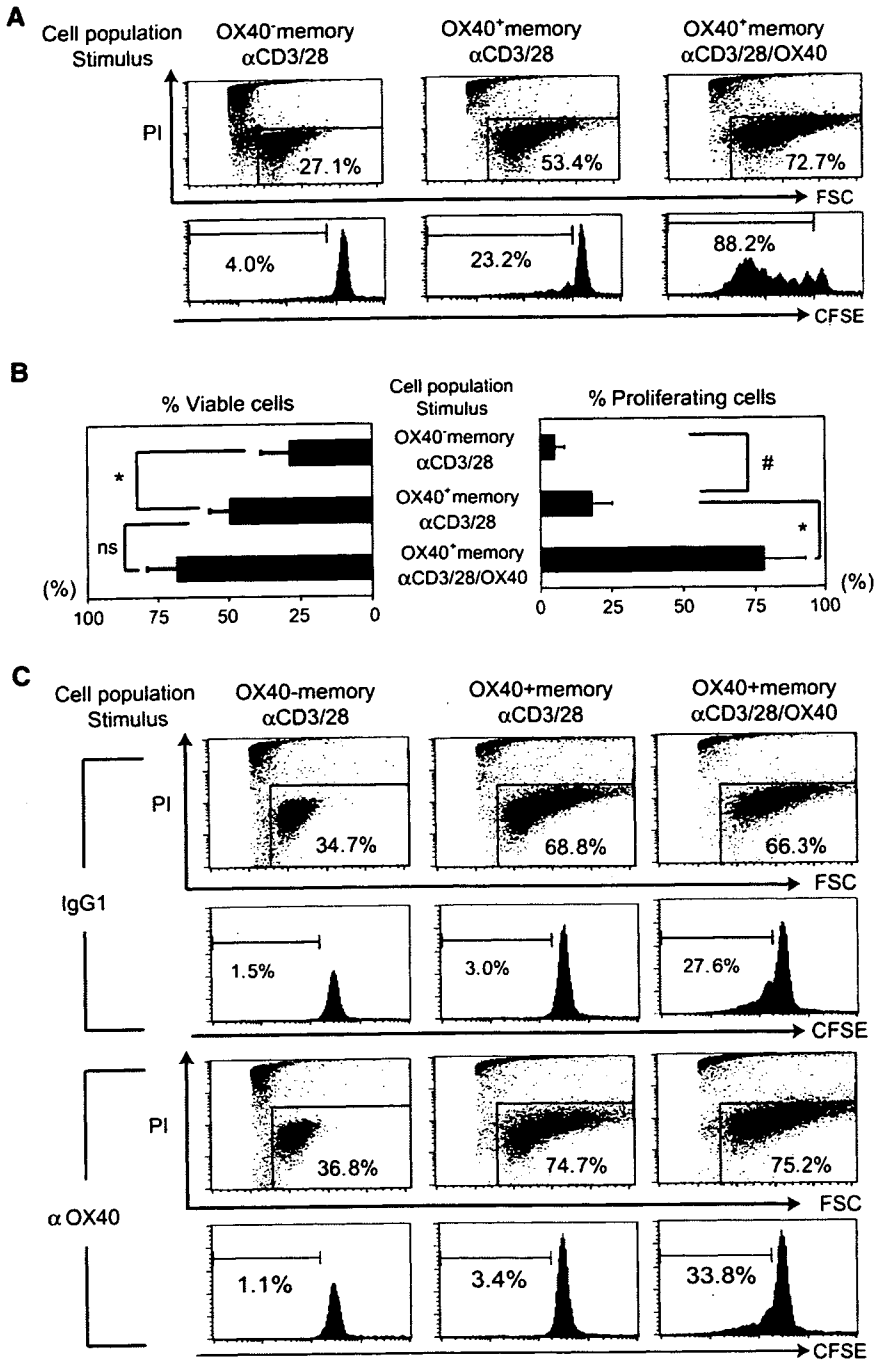

Figure 5. The presence of OX40-mediated signaling during antigenic stimulation results in explosive cell growth. (A,B) $OX40^+$ memory T cells and $OX40^-$ memory T cells from Allo HSCT recipients were labeled with CFSE, stimulated with αCD3/28 or αCD3/28/OX40 for 12 hours, washed, and cultured in growth medium for 4 days. (A) Upper dot plots show the PI-negative cells (viable cells) and lower histograms show the division profile of each cell population. The data shown are representative of 3 experiments. (B) The proportions of viable cells and proliferating cells are shown as means ± SD. $*P < .01$ $\#P < .02$ (C) $OX40^+$ memory T cells and $OX40^-$ memory T cells from HVs were labeled with CFSE, stimulated with αCD3/28 or αCD3/28/OX40 for 12 hours, washed, and then cultured in plates coated with mouse IgG1 (upper dot plots and histograms) or αOX40 Ab (lower dot plots and histograms) for 4 days. Dot plots show the PI staining and histograms show the division profile. The data shown are representative of 3 experiments.

Some of the latter cells return to the circulation as $OX40^+$ central memory T cells. In contrast, effector memory T cells that received antigenic stimulation would not return into circulation because they become effector cells and cannot survive long enough.

Surprisingly, IL-7, a cytokine critically involved in regulating the homeostasis of naïve and memory $CD4^+$ T cells, selectively upregulated the expression of OX40, enhanced IL-2-producing potential, and promoted antigen-independent proliferation in

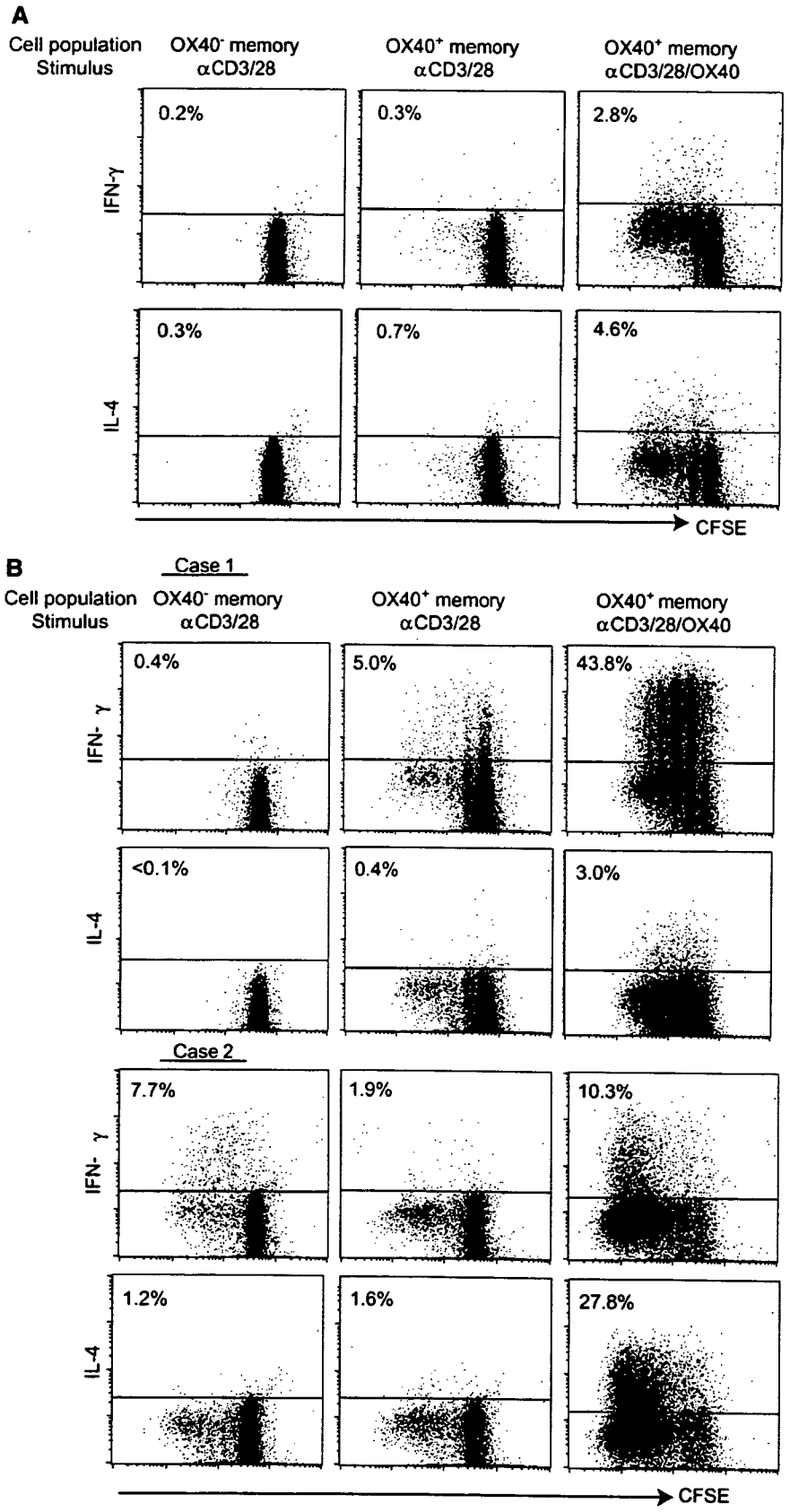

Figure 6. $CD4^+OX40^+$ T cells from Allo HSCT recipients differentiate into Th1 or Th2 effector cells in response to αCD3/28/OX40 stimulation. CFSE-labeled $OX40^+$ memory T cells and $OX40^-$ memory T cells from HVs (A) and Allo HSCT recipients (B) were stimulated with αCD3/28 or αCD3/28/OX40 for 12 hours, washed, cultured in medium for 4 days, and then restimulated with αCD3/28 for 6 hours. The division profile and cytokine production of the cells were then analyzed. (A) The dot plots are representative of 3 HVs. (B) The dot plots are representative of two Allo HSCT recipients.

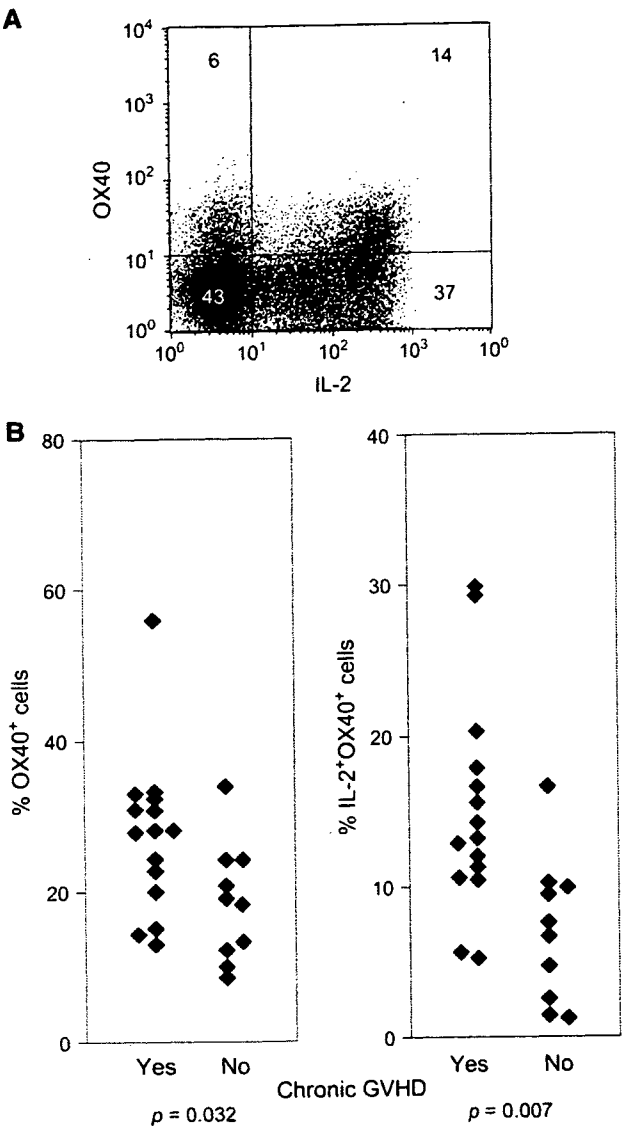

Figure 7. Allo HSCT recipients with cGVHD have much higher frequencies of IL-2-producing $CD4^+OX40^+$ T cells than Allo HSCT recipients without cGVHD. Peripheral blood mononuclear cells from Allo HSCT recipients were stimulated with PMA/Iono stimulation for 4 hours in the continuous presence of BFA. (A) A representative dot plot of surface OX40 and intracellular IL-2 is shown. The gates were set at the $CD4^+$ lymphocytes. (B) Comparison of the frequencies of OX40-expressing and IL-2-producing $OX40^+$ cells in the $CD4^+$ T cell populations of Allo HSCT recipients with (n = 15) and without (n = 10) cGVHD is shown.

$OX40^+$ memory cells. Interestingly, both $OX40^+$ memory and $OX40^-$ memory cells are positive for IL-7Rα, and IL-7 stimulation results in similar levels of STAT5 phosphorylation in both cell subsets. The selective action of IL-7 on $OX40^+$ memory cells implies that IL-7 in $OX40^+$ memory cells invokes a set of transcription factors that is not induced by IL-7 in $OX40^-$ memory cells. In other words, the expression of OX40 in $CD4^+$ T cells may guide IL-7 toward its cellular target. Although IL-7 is known to enhance T cell reconstitution after Allo HSCT [51,52], several reports have raised the concern that exogenous IL-7 administration to Allo HSCT recipients exacerbates GVHD [53,54]. In addition, endogenous IL-7, which is produced by the stromal cells in bone marrow, thymus, and lymph nodes [55], is suspected to ameliorate not only GVHD after Allo HSCT but also the rejection reaction after solid organ transplantation [56,57]. In any case, the significance of the increased sensitivity of $OX40^+$ memory cells to IL-7 will continue to be investigated.

We found that $CD4^+OX40^+$ T cells have a marked potential for proliferation and differentiation in vitro, especially if TCR ligation and costimulation through OX40 are provided simultaneously. Although we previously reported that OX40-mediated signaling on its own activates nuclear factor kappa B through a TNF receptor-associated factor-mediated pathway [58,59], sequential delivery of OX40-mediated signaling after the removal of TCR ligation did not result in cell growth or differentiation at all. Endl et al [60] have suggested that the expression of OX40 in vivo seems to be restricted to $CD4^+$ T cells that are exposed to high-affinity ligands. In addition, it takes at least a day for $CD4^+OX40^-$ memory T cells to express OX40 after antigenic stimulation in vitro. As activated dendritic cells have been shown to express OX40L [17,61], $CD4^+OX40^+$ T cells that enter the secondary lymphoid organs would have great advantage over $CD4^+OX40^-$ T cells in their clonal expansion and differentiation into effector memory T cells after their first encounters with APCs. In this study, there were some differences between $CD4^+OX40^+$ T cells of Allo HSCT recipients and those of HVs. Although $CD4^+OX40^+$ T cells of Allo HSCT recipients explosively proliferated and functionally differentiated in response to αCD3/28/OX40 stimulation, those of HVs did not (Figures 5A, the upper half of Figure 5C, and Figure 6). As the lymphopenic conditions seen in the Allo HSCT recipients promote the production of IL-7 [62], $CD4^+OX40^+$ T cells from Allo HSCT recipients might already have more of the IL-7-mediated signal than the $CD4^+OX40^+$ T cells from HVs before sampling. In addition, it is interesting that $OX40^+$ memory cells of some patients differentiated mainly into Th1-typed cells, whereas others showed Th2-biased differentiation.

Although the $CD4^+OX40^+$ T cell population contains cells that produce a large amount of IL-2, it also includes cells without IL-2-producing capacity. T cells with IL-2-producing capacity have been reported to actively proliferate and differentiate in vivo [63,64]. These results are meaningful when taken together with our finding that the frequency of IL-2-producing $CD4^+OX40^+$ T cells is much higher in Allo HSCT recipients with cGVHD than those without it. Recent studies on murine GVHD models have suggested

that donor-derived alloreactive T cells are activated in secondary lymphoid tissues before they migrate into target organs and cause tissue damage [65,66]. Although further studies are needed, the findings that CD4$^+$OX40$^+$ memory T cells with IL-2-producing capacity have increased sensitivity to IL-7 and can home to lymphoid organs and easily expand and differentiate into effector cells in response to antigenic stimulation, suggesting that they might have a role to play in the process of development and maintenance of cGVHD.

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bjh research paper

Clinical significance of nuclear non-phosphorylated beta-catenin in acute myeloid leukaemia and myelodysplastic syndrome

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Summary

Wnt signaling activates the canonical pathway and induces the accumulation of non-phosphorylated beta-catenin (NPBC) in the nucleus. Although this pathway plays an important role in the maintenance of haematopoietic stem cells as well as in oncogenesis, the significance of nuclear NPBC remains unclear in malignant haematopoiesis. This study examined the expression of nuclear NPBC in bone marrow specimens from 54 and 44 patients with *de novo* acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS), respectively. On immunohistochemistry with an anti-NPBC antibody, the nuclei were positively stained in 22 and 18 of AML and MDS specimens, respectively. Staining of nuclear NPBC was associated with AML subtypes (M6 and M7), low complete remission (CR) rate, and poor prognosis. Nuclear NPBC was also associated with a high score when using the International Prognostic Scoring System (IPSS) for MDS and with −7/−7q and complex karyotypes. These findings suggest that *in situ* detection of nuclear NPBC by immunohistochemistry could provide new insights into the pathogenesis and prognosis of AML and MDS.

Keywords: beta-catenin, non-phosphorylated beta-catenin, acute myeloid leukaemia, myelodysplastic syndrome, immunohistochemistry.

The Wnt/beta-catenin pathway is involved in the self-renewal and proliferation of haematopoietic stem cells (Reya *et al*, 2003; Willert *et al*, 2003). Signaling is initiated by binding of Wnt proteins to transmembrane receptors of the Frizzeled family (Giles *et al*, 2003). In the absence of Wnt signals, a dedicated complex of proteins that includes the tumor suppressor gene product APC, axin, and glycogen synthase kinase-3-beta (GSK3-beta) phosphorylates specific serine and threonine residues within the N-terminal region of beta-catenin, which leads to the ubiquitination of beta-catenin and its degradation by proteasomes (Conacci-Sorrell *et al*, 2002; Noort *et al*, 2002; Staal *et al*, 2002; Giles *et al*, 2003). Wnt signals block GSK3beta activity, resulting in the accumulation of non-phosphorylated beta-catenin (NPBC), which is finally translocated to the nucleus (Noort *et al*, 2002; Staal *et al*, 2002). Nuclear NPBC interacts with T-cell transcription factor (TCF) and lymphoid enhancer factor (LEF), and it activates target genes such as *MYC* and *CCND1* (He *et al*, 1998; Tetsu & Maccormick, 1999). Therefore, nuclear NPBC is known to be oncogenic in many solid tumors (Bienz & Clevers, 2000; Polakis, 2000). Mutations of APC, beta-catenin, or axin, which are observed in various tumors, lead to stabilization of NPBC (Morin *et al*, 1997; Barker & Clevers, 2000).