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Impact of the Direction of HLA Mismatch on Transplantation Outcomes in Single Unrelated Cord Blood Transplantation

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A B S T R A C T

The impact of the direction of HLA mismatch (MM) on outcome in unrelated cord blood (UCB) transplantation has not yet been clarified. We conducted a retrospective study using national registry data on 2977 patients who underwent transplantation using a single UCB for leukemia or myelodysplastic syndrome. HLA matching was assessed by serologic data for HLA-A, -B, and -DR loci. The median age of the recipients at transplantation was 41 years (range, 0-82 years), and 2300 recipients (77%) were age ≥16 years. The 2-year overall survival rate was 0.46. The presence of MM only in the graft-versus-host direction or only in the host-versus-graft direction was not associated with overall mortality (hazard ratio [HR], 0.88; $P = .317$ and HR, 0.95; $P = .670$, respectively) compared with 1 bidirectional MM. This finding was consistent in both the child and adult cohorts. The presence of MM only in the graft-versus-host direction was associated with a lower incidence of nonrelapse mortality (HR, 0.65; $P = .040$), significant only in the child cohort. No MM category was associated with relapse. Our findings suggest that the direction of HLA MM does not have a significant impact on overall survival after UCB transplantation.

INTRODUCTION

Unrelated cord blood (UCB) has emerged as a promising alternative source of hematopoietic stem cells for adult and pediatric allogeneic hematopoietic cell transplantation [1-4], and the use of UCB transplantation (UCBT) has been rapidly increasing, particularly in the United States, Europe, and Japan. One advantage of using UCB as a hematopoietic stem cell source is that UCBT requires less stringent HLA matching compared with bone marrow or peripheral blood stem cell transplantation, making it easier to find candidate UCB units in UCB banks. One or 2 antigen/allele mismatches (MMs) in the HLA-A, -B, and -DR loci between a UCB unit and recipient are acceptable without ex vivo T cell depletion methods, and the clinical outcome of transplantation using a 0-2 antigen/allele-mismatched UCB unit was almost comparable to that from an HLA allele-matched unrelated donor [1-3].

Although the number of HLA MMs between a UCB unit and a recipient is usually counted without considering the MM direction, the effect of the immune reaction caused by HLA MM differs according to whether the MM is in the graft-versus-host (GVH) or host-versus-graft (HVG) direction. A mismatched antigen in the GVH direction can be a major target for donor T cells and can cause graft-versus-host disease (GVHD), whereas a mismatched antigen in the HVG direction can be a major target for the remaining recipient T cells and can lead to graft rejection. In related transplantation, the presence of HLA MMs in the GVH direction is associated with a higher incidence of GVHD, whereas the

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presence of HLA MMs in the HVG direction is associated with a higher incidence of rejection [5-7]. Therefore, from a biological perspective, the impact of HLA MM should be discussed separately according to the direction of MM. However, because most patients have an equal number of MMs in the GVH and HVG directions (bidirectional MM), studying an adequate number of patients to evaluate an MM imbalance in the GVH and HVG directions has proven difficult.

The few studies that have evaluated the impact of the HLA MM direction on UCBT outcome have reported inconsistent results [8-10]. Matsuno et al. [8] reported that an HLA MM in the GVH direction was associated with lower incidence of neutrophil engraftment. In contrast, Stevens et al. [9] showed that UCBT with an MM only in the GVH direction was associated with a lower incidence of nonrelapse mortality (NRM) and overall mortality compared with UCBT with an 1 bidirectional MM, whereas UCBT with an MM only in the HVG direction was associated with a lower incidence of neutrophil engraftment and a higher incidence of relapse.

To clarify the significance of the direction of HLA MM on transplantation outcomes, we conducted a retrospective study using national registry data in 2977 patients who underwent a single UCBT.

METHODS

Data Collection

Data for 2987 patients with acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), and chronic myelogenous leukemia (CML) who underwent a first transplantation using a single UCB unit between January 1, 1998, and December 31, 2009, were obtained from the Transplant Registry Unified Management Program (TRUMP) [11], in which all UCBTs are registered through the Japan Cord Blood Bank Network (JCBBN), a national network of all 11 cord blood banks in Japan. Ten patients lacking data on survival status or survival date were excluded. A total of 2977 patients met the criteria for study inclusion. The study design was approved by the TRUMP Data Management Committee and the Institutional Review Board of Saitama Medical Center, Jichi Medical University, where this study was organized.

Histocompatibility

Histocompatibility data for the HLA-A, -B, and -DR loci were obtained from reports collected from the institution at which the transplantation was performed or cord blood banks. HLA typing methods have been described previously [12]. To reflect current practice in Japan, HLA matching was assessed by serologic data for HLA-A, -B, and -DR loci. A secondary analysis using antigen level data for HLA-A, -B and available allele level data for HLA-DRB1 was also performed to compare our data with previously published data from the United States and Europe. HLA-DRB1 allele information was available in 84% of patients (2498 of 2977). Among these patients, 62% had the same number of MMs at HLA-DRB1 loci at either the antigen or allele level. An HLA MM in the GVH direction was defined as when the recipient's antigens or alleles were not shared by the donor, and an MM in the HVG direction was defined as when the donor's antigens or alleles were not shared by the recipient.

Endpoints

The primary study endpoint was overall survival (OS). Other endpoints assessed were relapse, NRM, neutrophil and platelet engraftment, grade II-IV or III-IV acute GVHD, and chronic GVHD. Neutrophil recovery was defined as an absolute neutrophil count exceeding 0.5×10^9/L for 3 consecutive days after UCBT. Platelet recovery was defined as an absolute platelet count exceeding 50×10^9/L without platelet transfusion. The physicians who performed transplantation at each center diagnosed and graded acute and chronic GVHD according to traditional criteria [13,14]. The incidence of acute GVHD was evaluated in patients who engrafted, and that of chronic GVHD was evaluated in patients who engrafted and survived for more than 100 days.

Statistical Analysis

The probability of OS was estimated according to the Kaplan–Meier method and the groups were compared using the log-rank test. The probabilities of relapse, NRM, neutrophil and platelet engraftment, and acute and chronic GVHD were estimated based on cumulative incidence curves [15]. Competing events were death without relapse for relapse, relapse for NRM, death without engraftment for neutrophil and platelet engraftment, and death or relapse without GVHD for acute and chronic GVHD. The groups were compared using Gray's test [16]. The Cox proportional hazards model was used to evaluate the effect of confounding variables on OS, and the Fine and Gray proportional hazards model was used for the other endpoints [17]. Based on the report by the Center for International Blood and Marrow Transplant Research, we classified the conditioning regimens as myeloablative if total body irradiation >8 Gy, oral busulfan ≥9 mg/kg, i.v. busulfan ≥7.2 mg/kg, or melphalan >140 mg/m^2 was used in the conditioning regimen; otherwise, the conditioning regimen was classified as reduced intensity [18]. For patients with insufficient data regarding dosages of the agents used in the conditioning regimen, we used the information on conditioning intensity (myeloablative or reduced intensity) reported by the treating clinicians. We defined AML and ALL in first or second remission, CML in first or second chronic phase or accelerated phase, and MDS with refractory anemia or refractory anemia with ringed sideroblasts as standard risk, and all other conditions as high risk.

The following possible confounding variables were considered: recipient age group (0-5 years, 6-15 years, 16-49 years, or ≥50 years at transplantation), matching of ABO blood type between the recipient and UCB (match or major, minor, or bidirectional MM), recipient sex, sex MM between recipient and UCB (match, male donor–female recipient, or female donor–male recipient), disease (AML, ALL, CML, or MDS), disease status before transplantation (standard or high risk), type of conditioning regimen (myeloablative or reduced intensity), type of GVHD prophylaxis (calcineurin inhibitor plus methotrexate, calcineurin inhibitor only, others), and year of transplantation (1998-2004 or 2005-2009). Factors other than HLA MM and total nucleated cell (TNC) dose category were selected in a stepwise manner from the model with a variable retention criterion of $P < .05$. HLA MM and TNC dose category (≥10.0, 5.0-9.9, 2.5-4.9, 2.0-2.4, and <2.0 $\times 10^7$/kg) were then added to the final model. All tests were 2-sided, and a P value <.05 was considered statistically significant. All statistical analyses were performed with Stata version 12 (StataCorp, College Station, TX) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria) [19]. More precisely, EZR is a modified version of R commander (version 1.6-3) designed to add statistical functions used frequently used in biostatistics.

RESULTS

Patient Characteristics

Table 1 summarizes patient and transplant characteristics. The median age of the recipients at transplantation was 41 years (range, 0-82 years), and 2300 patients (77%) were age ≥16 years. Diagnoses for transplantation were AML in 1606 patients, ALL in 893, CML in 135, and MDS in 343. Half of the patients had standard-risk disease. UCBT was performed between 1998 and 2004 in 1153 patients (39%) and between 2005 and 2009 in 1824 patients (61%). The combination of a calcineurin inhibitor (tacrolimus or cyclosporine) and methotrexate was used in 62% of patients, whereas a calcineurin inhibitor alone was used in 22% of patients.

Some 40% of patients received a UCB unit containing <2.5 $\times 10^7$/kg TNCs, and 45% received a UCB unit containing 2.5-4.9 $\times 10^7$/kg TNCs. Roughly 12% of patients received ≥5.0 $\times 10^7$/kg TNCs, but 93% of these patients were age <16 years. Median body weight was 17 kg (range, 4-68 kg) for the children and 55 kg (range, 24-165 kg) for the adults. HLA MM was categorized as follows: HLA match in both the GVH and HVG directions (GVH 0/HVG 0 MM group; n = 273 [9%]), 1-2 antigen MMs in the GVH direction but 0 MMs in the HVG direction (GVH 1-2/HVG 0 MM group; n = 150 [5%]), 1-2 antigen MMs in the HVG direction but 0 MM in the GVH direction (GVH 0/HVG 1-2 MM group; n = 136 [5%]), 1 antigen MM in both the GVH and HVG directions at the same locus (GVH 1/HVG 1 MM group; n = 716 [24%]), 2 antigen MMs in both the GVH and HVG directions (GVH 2/HVG 2 MM group; n = 1170 [39%]), 2 antigen MMs in the GVH direction and 1 antigen MM in the HVG direction (GVH 2/HVG 1 MM group; n = 231 [8%]), 1 antigen MM in the GVH direction and

Table 1
Patient Characteristics

Characteristic	Total	Children (0-15 Years)	Adults (16+ Years)
Recipient age at UCBT, years, median (range)	41 (0-82)	5 (0-15)	49 (16-82)
Recipient age at UCBT, years, n (%)			
0-9	511 (17)	511 (75)	0 (0)
10-19	272 (9)	166 (25)	106 (5)
20-29	287 (10)	0 (0)	287 (12)
30-39	371 (12)	0 (0)	371 (16)
40-49	422 (14)	0 (0)	422 (18)
50-59	625 (21)	0 (0)	625 (27)
≥60	489 (16)	0 (0)	489 (21)
ABO matching, n (%)			
Match	994 (33)	248 (37)	746 (32)
Minor	815 (27)	174 (26)	641 (28)
Major	704 (24)	149 (22)	555 (24)
Bidirectional	458 (15)	104 (15)	354 (15)
Missing	6 (0)	2 (0)	4 (0)
Recipient sex, n (%)			
Female	1316 (44)	305 (45)	1011 (44)
Male	1661 (56)	372 (55)	1289 (56)
Donor–recipient sex match, n (%)			
Match	1157 (39)	290 (43)	867 (38)
Male donor and female recipient	635 (21)	153 (23)	482 (21)
Female donor and male recipient	768 (26)	172 (25)	596 (26)
Missing	417 (14)	62 (9)	355 (15)
Diagnosis, n (%)			
AML	1606 (54)	234 (35)	1372 (60)
ALL	893 (30)	391 (58)	502 (22)
CML	135 (5)	11 (2)	124 (5)
MDS	343 (12)	41 (6)	302 (13)
Disease risk at UCBT, n (%)			
Standard risk	1385 (47)	423 (62)	962 (42)
High risk	1450 (49)	226 (33)	1224 (53)
Missing	142 (5)	28 (4)	114 (5)
Conditioning regimen, n (%)			
Myeloablative	1980 (67)	585 (86)	1395 (61)
Reduced intensity	986 (33)	86 (13)	900 (39)
Missing	11 (0)	6 (1)	5 (0)
In vivo T cell depletion (ATG or alemtuzumab), n (%)			
No	2935 (99)	665 (98)	2270 (99)
Yes	42 (1)	12 (2)	30 (1)
GVHD prophylaxis, n (%)			
CSA only	250 (8)	59 (9)	191 (8)
TAC only	407 (14)	27 (4)	380 (17)
CSA + MTX	1105 (37)	209 (31)	896 (39)
TAC + MTX	755 (25)	241 (36)	514 (22)
CSA + MMF	104 (3)	0 (0)	104 (5)
TAC + MMF	148 (5)	6 (1)	142 (6)
CSA + corticosteroid	87 (3)	67 (10)	20 (1)
TAC + corticosteroid	34 (1)	26 (4)	8 (0)
Other	66 (2)	33 (5)	33 (1)
Missing	21 (1)	9 (1)	12 (1)
Year of UCBT, n (%)			
1998-2004	1153 (39)	389 (57)	764 (33)
2005-2009	1824 (61)	288 (43)	1536 (67)
TNC dose when frozen, n (%)			
$\geq 10.0 \times 10^7$/kg	99 (3)	99 (15)	0 (0)
$5.0\text{-}9.9 \times 10^7$/kg	259 (9)	234 (35)	25 (1)
$2.5\text{-}4.9 \times 10^7$/kg	1344 (45)	268 (40)	1076 (47)
$2.0\text{-}2.4 \times 10^7$/kg	924 (31)	44 (6)	880 (38)
$<2.0 \times 10^7$/kg	275 (9)	21 (3)	254 (11)
Missing	76 (3)	11 (2)	65 (3)
Weight, kg, median (range)	52 (4-165)	17 (4-68)	55 (24-165)
HLA MM			
0 MM	273 (9)	144 (21)	129 (6)
1-2 MM/GVH only	150 (5)	45 (7)	105 (5)
1-2 MM/rejection only	136 (5)	39 (6)	97 (4)
1 bidirectional MM	716 (24)	314 (46)	402 (17)
2 bidirectional MM	1170 (39)	98 (14)	1072 (47)
2 MM: bidirectional + GVHD	231 (8)	16 (2)	215 (9)
2 MM: bidirectional + rejection	264 (9)	19 (3)	245 (11)
2 MM: GVHD + rejection	37 (1)	2 (0)	35 (2)

ATG indicates antithymocyte globulin; CSA, cyclosporine; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus; 0 MM, HLA match in both the GVH and HVG directions; 1-2 MM/GVH only, antigen MMs in the GVH direction and 0 MMs in the HVG direction; 1-2 MM/rejection only, 1 or 2 antigen MMs in the HVG direction and no MMs in the GVH direction; 1 bidirectional MM, 1 antigen MM in both the GVH and HVG directions at the same locus; 2 bidirectional MM, 2 antigen MMs in both the GVH and HVG directions; 2 MM: bidirectional + GVHD, 2 antigen MMs in the GVH direction and 1 antigen MM in the HVG direction; 2 MM: bidirectional + rejection, 1 antigen MM in the GVH direction and 2 antigen MMs in the HVG direction; 2 MM: GVHD + rejection, 1 antigen MM in the GVH direction at one locus and 1 antigen MM in the HVG direction at another locus.

2 antigen MMs in the HVG direction (GVH 1/HVG 2 MM group; n = 264 [9%]), and 1 antigen MM in the GVH direction at 1 locus and 1 antigen MM in the HVG direction at another locus (GVH 1/HVG 1 2-antigen MM group; n = 37 [1%]).

OS, Relapse, and NRM

The median follow-up period in survivors was 2.2 years (range, 0.0-11.1 years). The 2-year OS rate was 0.46 (95% confidence interval [CI], 0.44-0.48) (Figure 1). To clarify the impact of HLA MM in each vector, the GVH 1/HVG 1 MM group was considered the reference group in the multivariate analyses, in accordance with the approach of Stevens et al. [9], and the following hazard ratios (HRs) were adjusted for the other significant variables, including TNC dose category. The GVH 1-2/HVG 0 MM (HR, 0.88; 95% CI, 0.69-1.13; $P = .317$), the GVH 0/HVG 1-2 MM (HR, 0.95; 95% CI, 0.74-1.22; $P = .670$), and other groups were not associated with overall mortality compared with the GVH 1/HVG 1 MM group (Table 2 and Figure 1). The GVH 0/HVG 0 MM group

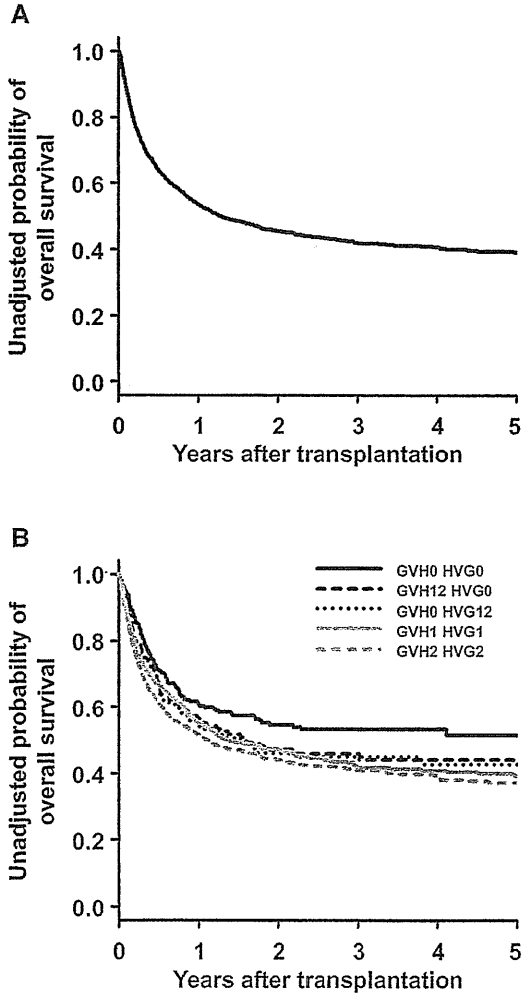

Figure 1. OS of total patients (A) and patients grouped according to HLA MM category (B). GVH0 HVG0, HLA match in both the GVH and HVG directions; GVH12 HVG0, antigen MMs in the GVH direction and 0 MMs in the HVG direction; GVH0 HVG12, 1 or 2 antigen MMs in the HVG direction and 0 MM in the GVH direction; GVH1 HVG1, 1 antigen MM in both the GVH and HVG directions at the same locus; GVH2 HVG2, 2 antigen MMs in both the GVH and HVG directions.

Table 2
Overall Mortality

HLA MM Category	Total*			Child			Adult		
	Number	HR 95% CI	*P* Value	Number	HR 95% CI	*P* Value	Number	HR 95% CI	*P* Value
0 MM	273	0.79 (0.64-0.97)	.025	144	0.74 (0.53-1.04)	.079	129	0.82 (0.63-1.06)	.134
1-2 MM/GVH only	150	0.88 (0.69-1.13)	.317	45	0.73 (0.44-1.20)	.208	105	0.92 (0.69-1.22)	.560
1-2 MM/rejection only	136	0.95 (0.74-1.22)	.670	39	0.85 (0.49-1.46)	.557	97	0.96 (0.72-1.29)	.796
1 bidirectional MM	716	1.00	Reference	314	1.00	Reference	402	1.00	Reference
2 bidirectional MM	1170	0.90 (0.79-1.03)	.122	98	0.88 (0.61-1.26)	.480	1072	0.89 (0.76-1.03)	.118
2 MM: bidirectional + GVHD	231	0.97 (0.79-1.18)	.737	16	1.10 (0.55-2.20)	.785	215	0.94 (0.76-1.17)	.594
2 MM: bidirectional + rejection	264	0.93 (0.77-1.13)	.481	19	2.08 (1.19-3.63)	.011	245	0.87 (0.71-1.08)	.208
2 MM: GVHD + rejection	37	0.55 (0.35-0.87)	.012	2			35	0.54 (0.33-0.87)	.012

* Other significant variables were recipient age group, 0-5 years (reference, 1.00), 6-15 years (HR, 0.93; 95% CI, 0.69-1.24; $P = .603$), 16-49 years (HR, 1.32; 95% CI, 1.00-1.75; $P = .053$), ≥50 years (HR, 1.96; 95% CI, 1.48-2.60; $P < .001$); recipient sex, female (reference, 1.00), male (HR, 1.16; 95% CI, 1.05-1.28; $P = .005$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.42; 95% CI, 2.16-2.71; $P < .001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 1.30; 95% CI, 1.15-1.46; $P < .001$), others (HR, 1.13; 95% CI, 0.98-1.31; $P = .091$), year of transplantation, 1998-2004 (reference, 1.00), 2005-2009 (HR, 0.83; 95% CI, 0.75-0.93; $P = .001$).

was associated with lower overall mortality compared with the GVH 1/HVG 1 MM group (HR, 0.79; 95% CI, 0.64-0.97; $P = .025$); however, in both the child and adult cohorts, the association was not significant, owing in part to a lack of statistical power. The GVH 1/HVG 1 2-antigen MM group, which was represented mostly in the adult cohort, was associated with lower overall mortality compared with the GVH 1/HVG 1 MM group (HR, 0.55; 95% CI, 0.35-0.87; $P = .012$).

We performed an additional analysis according to the HLA matching criteria used in the United States and Europe (HLA-A and -B for antigen level and -DRB1 for allele level) (Supplemental Table 1). Consistent with the result obtained using our criteria (HLA-A, -B, and -DR for antigen level), there were no differences in the impact of the MM direction (GVH or HVG) on OS. The difference in OS between the GVH 0/HVG 0 MM and GVH 1/HVG 1 MM groups was not significant in this analysis.

The cumulative incidence rates of relapse and NRM at 2 years were 0.34 (95% CI, 0.32-0.36) and 0.26 (95% CI, 0.24-0.27), respectively. There was no difference in the incidence of relapse between the GVH 1/HVG 1 MM and any other MM group (Table 3 and Figure 2). The GVH 1-2/HVG 0 MM group was significantly associated with lower NRM compared with the GVH 1/HVG 1 MM group (HR, 0.65; 95% CI, 0.44-0.98; $P = .040$) (Table 3 and Figure 2), but only in the child cohort (child, $P = .048$; adult, $P = .215$).

Because our cohorts were mainly adults, and most adults received a TNC dose of 2.0-4.9 $\times$ 10^7/kg, we performed an additional analysis in the subset of adults who received a TNC dose of 2.0-2.4 $\times$ 10^7/kg or 2.5-4.9 $\times$ 10^7/kg (Supplemental Table 2). In the subset of adults who received a TNC dose of 2.0-2.4 $\times$ 10^7/kg, compared with the GVH 1/HVG 1 MM group, the GVH 0/HVG 0 MM group was associated with lower overall mortality ($P = .027$) and NRM ($P = .007$) and a higher incidence of relapse ($P = .028$), and the GVH 2/HVG 2 MM group was associated with lower overall mortality ($P = .001$) and NRM ($P = .008$). The GVH 1-2/HVG 0 MM group was significantly associated with lower NRM compared with the GVH 1/HVG 1 MM group ($P = .033$). In the subset of adults who received a TNC dose of 2.5-4.9 $\times$ 10^7/kg, no HLA MM group was associated with overall mortality, relapse, or NRM, except for lower overall mortality in the GVH 1/HVG 1 2-antigen MM group compared with the GVH 1/HVG 1 MM group ($P = .046$).

Neutrophil and Platelet Engraftment

The cumulative incidence rates of neutrophil and platelet engraftment in our study cohort were 0.76 (95% CI, 0.74-0.77) and 0.57 (95% CI, 0.55-0.59), respectively. The GVH 1-2/HVG 0 MM group was marginally associated with better neutrophil and platelet engraftment kinetics compared with the GVH 1/HVG 1 MM group (neutrophil engraftment: HR, 1.18; 95% CI, 0.98-1.42; $P = .081$; platelet engraftment: HR, 1.23; 95% CI, 1.00-1.51; $P = .053$) (Table 4 and Figure 3). The impact on neutrophil engraftment was significant only in the adult cohort (child, $P = .496$; adult, $P = .045$).

Acute and Chronic GVHD

In all engrafted patients, the cumulative incidence rates of grade II-IV and III-IV acute GVHD were 0.45 (95% CI, 0.43-0.47) and 0.15 (95% CI, 0.14-0.17), respectively. The GVH 0/HVG 0 MM group was significantly associated with a lower incidence of grade II-IV acute GVHD compared with the GVH 1/HVG 1 MM group (HR, 0.70; 95% CI, 0.54-0.90; $P = .006$) (Supplemental Table 3 and Figure 4), but only in the child cohort (child, $P = .002$; adult, $P = .506$). The GVH 0/HVG 0 MM group was marginally associated with a lower incidence of chronic GVHD compared with the GVH 1/HVG 1 MM group (HR, 0.72; 95% CI, 0.51-1.00; $P = .050$).

DISCUSSION

This nationwide retrospective study that included a large number of both pediatric and adult patients allowed us to consider an adequate number of patients who underwent UCBT with an HLA MM only in the GVH direction or only in the HVG direction, and to analyze the impact of an MM in the GVH or HVG direction on clinical outcomes after a single UCBT. Neither the GVH 1-2/HVG 0 MM group nor the GVH 0/HVG 1-2 MM group was associated with overall mortality compared with the GVH 1/HVG 1 MM group. The point estimates of HRs of the GVH 1-2/HVG 0 MM and GVH 0/HVG 1-2 MM groups compared with the GVH 1/HVG 1 MM group were similar and both <1 (HR, 0.88 and 0.95, respectively), suggesting that HLA MMs in the GVH and HVG directions post-UCBT do not have different effects on OS. This finding does not support the conclusion of Stevens et al. [9], who recommended using UCB units with an HLA MM only in the GVH direction and avoiding units with an HLA MM only in the HVG direction.

Table 3
Relapse and NRM

HLA MM category	Relapse*			NRM†		
	Number	HR 95% CI	*P* Value	Number	HR 95% CI	*P* Value
0 MM	258	1.07 (0.84-1.37)	.560	258	0.74 (0.53-1.02)	.063
1-2 MM/GVH only	147	1.20 (0.90-1.59)	.215	147	0.65 (0.44-0.98)	.040
1-2 MM/rejection only	131	1.18 (0.84-1.64)	.338	131	0.81 (0.55-1.19)	.292
1 bidirectional MM	667	1.00	Reference	667	1.00	Reference
2 bidirectional MM	1106	0.99 (0.83-1.19)	.930	1106	0.88 (0.72-1.07)	.191
2 MM: bidirectional + GVHD	217	1.00 (0.76-1.33)	.979	217	0.81 (0.60-1.10)	.184
2 MM: bidirectional + rejection	243	1.27 (0.99-1.63)	.060	243	0.66 (0.49-0.91)	.010
2 MM: GVHD + rejection	36	0.64 (0.32-1.24)	.184	36	0.61 (0.32-1.16)	.131

* Other significant variables were recipient age group, 0-5 years (reference, 1.00), 6-15 years (HR, 0.61; 95% CI, 0.44-0.84; $P = .002$), 16-49 years (HR, 0.71; 95% CI, 0.52-0.97; $P = .030$), ≥50 years (HR, 0.72; 95% CI, 0.52-0.98; $P = .040$); diagnosis, AML (reference, 1.00), ALL (HR, 1.11, 95% CI, 0.94-1.30, $P = .210$), CML (HR, 1.33, 95% CI, 0.99-1.79, $P = .059$), MDS (HR, 0.67, 95% CI, 0.51-0.87, $P = .003$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.93; 95% CI, 2.54-3.39; $P < .001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 0.72; 95% CI, 0.61-0.86; $P < .001$), others (HR, 0.87; 95% CI, 0.71-1.05; $P = .145$).

† Other significant variables were recipient age group, 0-5 years (reference, 1.00), 6-15 years (HR, 1.44; 95% CI, 0.90-2.30; $P = .128$), 16-49 years (HR, 2.04; 95% CI, 1.29-3.22; $P = .002$), ≥50 years (HR, 3.52; 95% CI, 2.24-5.52; $P < .001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 1.90; 95% CI, 1.60-2.26; $P < .001$), others (HR, 1.42; 95% CI, 1.14-1.75; $P = .001$), year of transplantation, 1998–2004 (reference, 1.00), 2005-2009 (HR, 0.71; 95% CI, 0.61-0.83; $P < .001$).

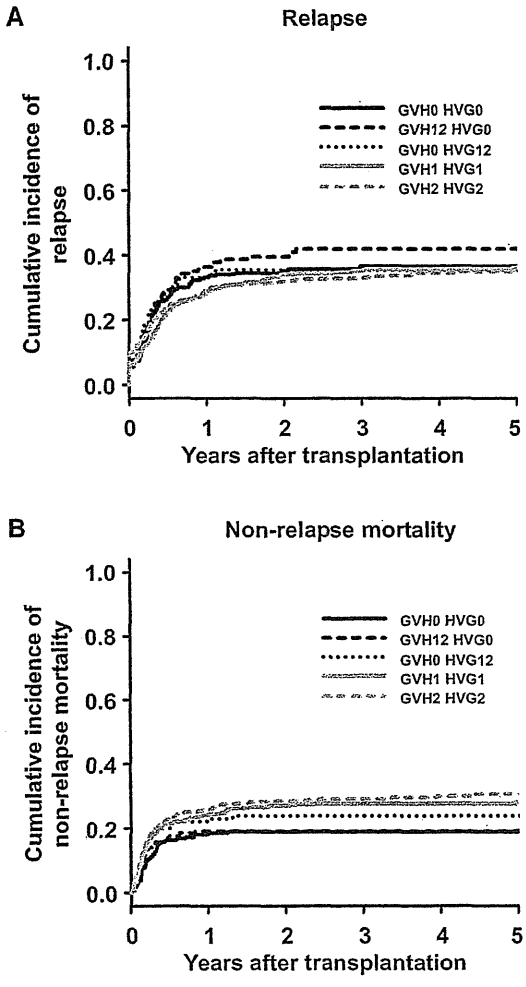

Figure 2. Relapse and NRM.

Several differences in patient background between the study of Stevens et al. [9] and the present study warrant clarification. The first difference is in the age distribution of patients. Stevens et al.'s series included 907 pediatric patients age <16 years and 295 adult patients; in contrast, our series included 677 pediatric patients and 2300 adult patients, which can provide useful information for both pediatric and adult transplant physicians. Since the cell doses of UCB units in child and adult cohorts are significantly different, which may affect the impact of HLA MM, we performed stratified analyses in the child and adult cohorts. Our results consistently showed that the direction of MM had no apparent impact on overall mortality in either cohort. Consistent with the results of Stevens et al. [9], the GVH 1-2/HVG 0 MM group was associated with lower NRM in the child cohort, but this advantage was offset by a higher incidence of relapse in this cohort. A second difference between the 2 studies is in conditioning regimens. A myeloablative regimen was used in 92% of the patients in the Stevens et al. study, compared with 67% in our study. Consequently, we performed a separate analysis in the patients who received a myeloablative regimen and confirmed that the direction of MM had no apparent impact on overall mortality (data not shown).

The third difference between the 2 studies relates to GVHD prophylaxis. Cyclosporine and steroids were used as GVHD prophylaxis in 62% of the patients in the Stevens et al. study, but in only 3% of the patients (10% of the child cohort) in our study, which might have affected outcomes. The fourth difference is in the number of patients with an HLA MM only in the GVH direction or only in the HVG direction. The Stevens et al. study included 35 patients with a GVH 1-2/HVG 0 MM and 22 patients with a GVH 0/HVG 1-2 MM in the overall mortality analysis, compared with 150 and 136 patients, respectively, in our study. Finally, the level of HLA typing used to determine the number of HLA MMs differed between the 2 studies. In the present study, MMs in HLA-DR loci were counted at the antigen level in accordance with current practice in Japan, whereas Stevens et al. counted HLA-DRB1 MMs at the allele level. Consequently, we performed an additional analysis using the same HLA matching criteria as in previous studies from the United States and Europe (HLA-A and -B for antigen level and -DRB1 for allele level), and reached a similar conclusion that an MM only in the GVH or only in the HVG direction had no impact on overall mortality.

Table 4
Neutrophil and Platelet Engraftment

HLA MM Category	Neutrophil Engraftment*			Platelet Engraftment†		
	Number	HR 95% CI	*P* Value	Number	HR 95% CI	*P* Value
0 MM	272	1.03 (0.88-1.20)	.718	272	1.06 (0.88-1.27)	.559
1-2 MM/GVH only	149	1.18 (0.98-1.42)	.081	149	1.23 (1.00-1.51)	.053
1-2 MM/rejection only	136	1.01 (0.82-1.26)	.899	136	0.84 (0.66-1.07)	.164
1 bidirectional MM	716	1.00	Reference	714	1.00	Reference
2 bidirectional MM	1167	0.98 (0.87-1.09)	.672	1166	0.96 (0.85-1.10)	.590
2 MM: bidirectional + GVHD	231	0.91 (0.76-1.08)	.278	230	0.91 (0.74-1.13)	.406
2 MM: bidirectional + rejection	264	0.86 (0.72-1.02)	.089	264	0.98 (0.80-1.19)	.816
2 MM: GVHD + rejection	37	1.40 (1.03-1.89)	.030	37	2.21 (1.46-3.33)	<.001

* Other significant variables were TNC category, 2.5-4.9 × 10^7/kg (reference, 1.00), ≥10.0 × 10^7/kg (HR, 1.76; 95% CI, 1.33-2.33; $P < .001$), 5.0-9.9 × 10^7/kg (HR, 1.26; 95% CI, 1.05-1.52; $P = .015$), 2.0-2.4 × 10^7/kg (HR, 0.87; 95% CI, 0.79-0.95; $P = .003$), <2.0 × 10^7/kg (HR, 0.82; 95% CI, 0.71-0.95; $P = .007$); diagnosis, AML (reference, 1.00), ALL (HR, 1.11, 95% CI, 1.00-1.22, $P = .040$), CML (HR, 0.87, 95% CI, 0.73-1.04, $P = .124$), MDS (HR, 0.88, 95% CI, 0.75-1.04, $P = .129$); disease risk, standard risk (reference, 1.00), high risk (HR, 0.74; 95% CI, 0.67-0.80; $P < .001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 1.16; 95% CI, 1.04-1.30; $P = .010$), others (HR, 1.09; 95% CI, 0.96-1.23; $P = .169$), year of transplantation, 1998-2004 (reference, 1.00), 2005-2009 (HR, 1.21; 95% CI, 1.11-1.33; $P < .001$).

† Other significant variables were TNC category, 2.5-4.9 × 10^7/kg (reference, 1.00), ≥10.0 × 10^7/kg (HR, 1.49; 95% CI, 1.09-2.03; $P = .013$), 5.0-9.9 × 10^7/kg (HR, 1.26; 95% CI, 1.01-1.57; $P = .040$), 2.0-2.4 × 10^7/kg (HR, 0.95; 95% CI, 0.84-1.06; $P = .365$), <2.0 × 10^7/kg (HR, 0.81; 95% CI, 0.67-0.97; $P = .022$); recipient age group, 0-5 years (reference, 1.00), 6-15 years (HR, 1.00; 95% CI, 0.79-1.25; $P = .971$), 16-49 years (HR, 0.99; 95% CI, 0.77-1.26; $P = .909$), ≥50 years (HR, 0.70; 95% CI, 0.55-0.90; $P = .006$); recipient sex, female (reference, 1.00), male (HR, 0.90; 95% CI, 0.82-0.99; $P = .034$); disease risk, standard risk (reference, 1.00), high risk (HR, 0.58; 95% CI, 0.53-0.64; $P < .001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 0.81; 95% CI, 0.71-0.91; $P = .001$), others (HR, 0.88; 95% CI, 0.76-1.01; $P = .074$), and year of transplantation, 1998-2004 (reference, 1.00), 2005-2009 (HR, 1.26; 95% CI, 1.14-1.40; $P < .001$).

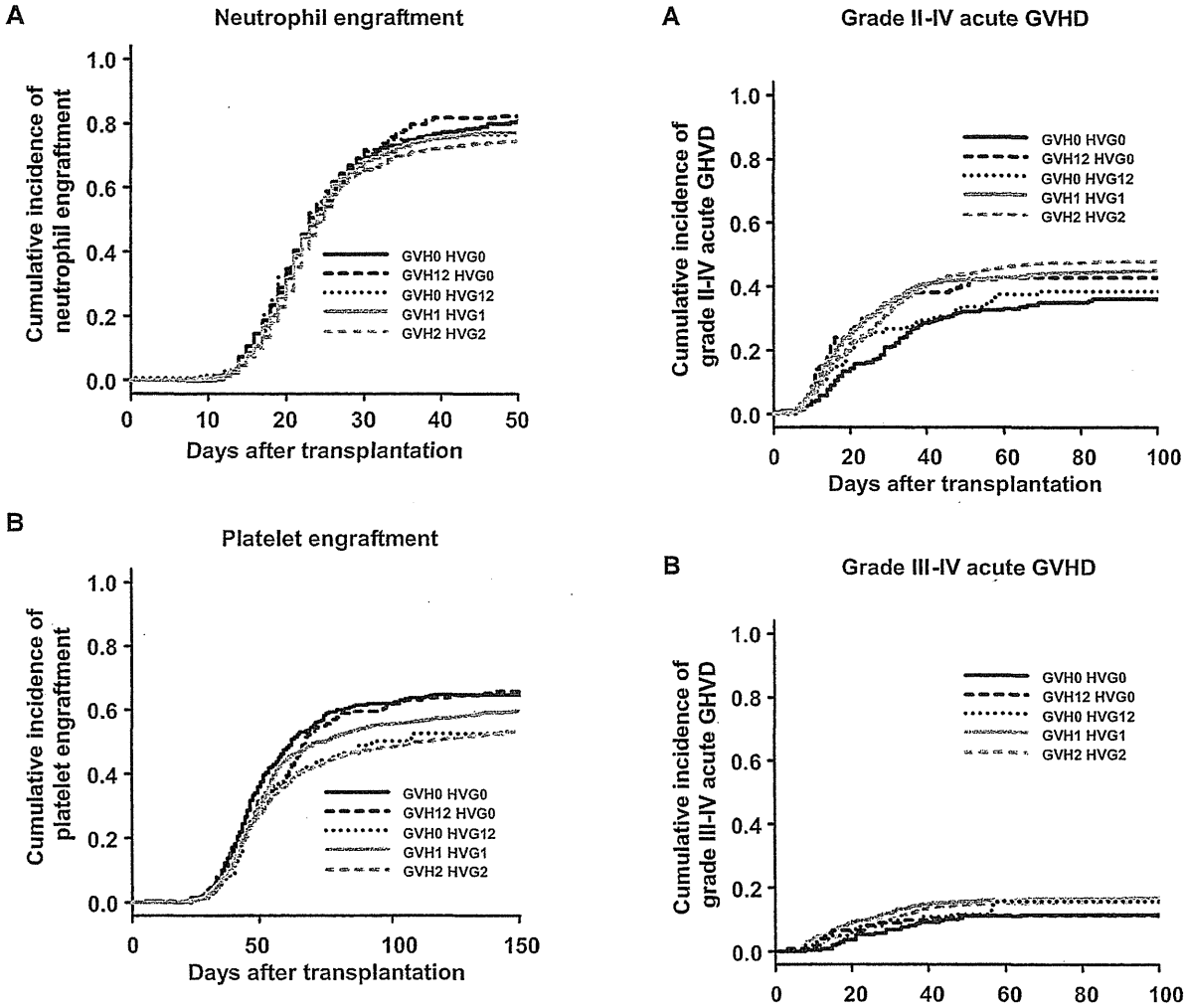

Figure 3. Neutrophil and platelet engraftment.

Figure 4. Acute and chronic GVHD.

Similar to Stevens et al. [9], we found a tendency for better neutrophil and platelet engraftment kinetics in the GVH 1-2/HVG 0 MM group. This finding suggests that an HLA MM in the GVH direction enhances engraftment by eradicating or suppressing the host residual immune cells responsible for the rejection or inhibition of donor cell engraftment. In contrast to our findings, Matsuno et al. [8] analyzed the impact of GVH/HVG MM on 152 patients who underwent a single UCBT in a single center, and found that the presence of a 2-antigen MM in the GVH direction was associated with slower and lower neutrophil engraftment compared with a 0- or 1-antigen MM in the GVH direction. Because Matsuno et al. used only a calcineurin inhibitor for GVHD prophylaxis in all of the patients in their cohort, we recategorized the HLA MM group according to HLA category (GVH 0-1/HVG 0-1 MM, GVH 0–1/HVG 2 MM, GVH 2/HVG 0-1 MM, and GVH 2/HVG 2 MM) and performed additional analyses in which patients were stratified according to GVHD prophylaxis (calcineurin inhibitor plus methotrexate, calcineurin inhibitor only, or other). Similar to the findings of Matsuno et al., an MM in the GVH direction was significantly associated with a lower incidence of engraftment in patients who received only a calcineurin inhibitor (data not shown). In contrast, an MM in the GVH direction was associated with a higher incidence of engraftment in patients who received a calcineurin inhibitor plus methotrexate. These findings suggest that the impact of HLA MM differs according to GVHD prophylaxis. A possible explanation for the different

effects of GVHD prophylaxis on engraftment is the high incidence of hemophagocytic syndrome (HPS) and pre-engraftment immune reaction in patients who received only a calcineurin inhibitor as GVHD prophylaxis [20,21]. Takagi et al. [20] reported HPS in 20 of 119 patients who underwent UCBT with mostly tacrolimus alone as GVHD prophylaxis, resulting in a high incidence of graft failure. Less-intensive GVHD prophylaxis may enhance the immune reaction caused by donor T cells that recognize the HLA MM antigen in the GVH direction in the early phase after transplantation, which could lead to HPS or similar conditions and decrease the rate of neutrophil engraftment. These findings demonstrate the need for a prospective study using uniform GVHD prophylaxis to further evaluate the impact of HLA MM on neutrophil engraftment.

This study has several limitations. First, the patients' heterogeneous backgrounds might have produced statistical bias, although we attempted to reduce this bias by adjusting the impact in the multivariate analyses. Second, the number of subjects in each HLA MM group category was limited. Nevertheless, the number of subjects in the GVH 1-2/HVG 0 and GVH 0/HVG 1-2 MM groups was much greater than that in previous studies [8,9]. Third, we might have underestimated the degree of HLA MM, given our incomplete allelic and HLA-C antigen information; for example, the group that had only an HLA MM in the GVH direction might have included an allelic MM in the HVG direction. A potential HLA-C antigen MM or KIR ligand MM also might have affected outcomes, but we did not evaluate HLA-C in the present study. The foregoing issues might have weakened the power of this study to detect differences.

In conclusion, our findings do not support a strategy for selecting UCB donors based on the direction of the HLA MM, although GVH 1-2/HVG 0 MMs may be associated with better neutrophil engraftment, particularly when a calcineurin inhibitor plus other immunosuppressive agents, such as methotrexate, are used for GVHD prophylaxis. The impact of HLA MMs in only the GVH direction remains to be clarified further under a uniform GVHD prophylaxis regimen.

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Authorship statement: J.K. and Y.K. designed the research, organized the project, and wrote the manuscript. J.K., Y.A., and Y.K. performed the statistical analysis and analyzed the data; K.K. and T.N.-I. collected data from JCBBN; and all of the authors interpreted the data, and reviewed and approved the final manuscript.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.09.017.

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Cell Stem Cell

Generation of Rejuvenated Antigen-Specific T Cells by Reprogramming to Pluripotency and Redifferentiation

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SUMMARY

Adoptive immunotherapy with functional T cells is potentially an effective therapeutic strategy for combating many types of cancer and viral infection. However, exhaustion of antigen-specific T cells represents a major challenge to this type of approach. In an effort to overcome this problem, we reprogrammed clonally expanded antigen-specific $CD8^+$ T cells from an HIV-1-infected patient to pluripotency. The T cell-derived induced pluripotent stem cells were then redifferentiated into $CD8^+$ T cells that had a high proliferative capacity and elongated telomeres. These "rejuvenated" cells possessed antigen-specific killing activity and exhibited T cell receptor gene-rearrangement patterns identical to those of the original T cell clone from the patient. We also found that this method can be effective for generating specific T cells for other pathology-associated antigens. Thus, this type of approach may have broad applications in the field of adoptive immunotherapy.

INTRODUCTION

T cells play a central role in acquired immunity and the configuration of systemic immunity against pathogens. In particular, cytotoxic T lymphocytes (CTLs) are major components of this systemic response to microorganisms, viral infections, and neoplasms (Greenberg, 1991; Zhang and Bevan, 2011). T cells initiate their proliferative and effector functions upon human leukocyte antigen (HLA)-restricted recognition of specific antigen peptides via T cell receptors (TCRs). This is greatly beneficial in enabling the selective recognition and eradication of target cells, and also in long-term immunological surveillance by long-lived memory T cells (Butler et al., 2011; Jameson and Masopust, 2009; MacLeod et al., 2010). However, viruses in chronic infection or cancers often hamper or escape the T cell immunity by decreasing the expression of molecules required for T cell recognition or by inhibiting antigen presentation (Virgin et al., 2009). In addition, continuous exposure to chronically expressed viral antigens or cancer/self-antigens can drive T cells into an "exhausted" state. This is characterized by loss of effector functions and the potential for long-term survival and proliferation, ultimately leading to the depletion of antigen-responding T cell pools (Klebanoff et al., 2006; Wherry, 2011).

The infusion of ex vivo-expanded autologous antigen-specific T cells is being developed clinically for T cell immunotherapy. However, up to now, highly expanded T cells have not proven to be particularly effective (June, 2007). This is in part explained by losses of function that occur during the ex vivo manipulation of patient autologous T cells. In another instance, genetic modification of antigen receptors is an ambitious but only partially successful way to add desired antigen specificity to nonspecific T cells (Morgan et al., 2006; Porter et al., 2011). The therapeutic effect also strongly depends on the extent of functional loss that occurs during the ex vivo manipulation of T cells and on the stability of exogenous antigen receptor expression specific to target molecules in the presence of the endogenous TCR genes (Bendle et al., 2010; Brenner and Okur, 2009).

For the purpose of overcoming these obstacles, the therapeutic potential of induced pluripotent stem cells (iPSCs) is being

explored. Embryonic stem cells (ESCs) or iPSCs have the capacity for self-renewal while maintaining pluripotency (Takahashi et al., 2007) and could potentially form the basis for the unlimited induction of antigen-specific juvenile T cells. However, there are challenges to this approach as well, given that methods for the differentiation and immunological education of ESCs and iPSCs, or indeed that of intermediate hematopoietic stem and/or progenitor cells, into fully matured functional human T cells are not well established (Timmermans et al., 2009). Reprogramming the nuclei of lymphocytes was historically performed for studying whether terminally differentiated or fully matured somatic cells could revert to a pluripotent state. The first demonstration of lymphocyte reprogramming employed somatic cell nuclear transfer in murine B and T cells, proving that terminally differentiated somatic cells were reprogrammable (Hochedlinger and Jaenisch, 2002). Reprogramming murine B cells into pluripotent stem cells by iPSC technology also provided definite proof for fate reversibility in fully matured somatic cells (Hanna et al., 2008). From another point of view, nuclear reprogramming of lymphocytes is seen as having applications for regenerative medicine different than those for scientific research. The irreversible rearrangement of genes encoding immunoglobulins and TCRs was recognized solely as a genetic marker in somatic cell nuclear transfer and iPSC research. However, the preserved rearrangements in genomic DNA can also provide a blueprint of "educated" weapons for attacking cancers and pathogens in adoptive immunotherapy. Although several groups have reported the generation of T cell-derived iPSCs (T-iPSCs), their clinical applications have yet to be thoroughly explored (Brown et al., 2010; Loh et al., 2010; Seki et al., 2010; Staerk et al., 2010).

In the present study, we chose a T cell clone specific to an HIV type 1 (HIV-1) epitope of known structure to act as a generic representation of iPSC-mediated T cell regeneration. We successfully induced iPSCs from antigen-specific T cells and redifferentiated them into functional T cells. This may act as proof of concept for the application of "rejuvenated" T cells in treating various diseases. Crucial to this concept was that T-iPSCs retained the assembled "endogenous" TCR genes even after being subjected to nuclear reprogramming. Furthermore, redifferentiated T cells showed the same pattern of TCR gene arrangement as that in the original T cells. These features may therefore serve as the foundation for the reproduction of unlimited numbers of T cells that express desired TCRs conferring to antigen specificity.

RESULTS

Reprogramming an Antigen-Specific Cytotoxic T Cell Clone into Pluripotency

To establish T cell-derived iPSCs, we magnetically separated the $CD3^+$ T cell population from peripheral blood mononuclear cells (PBMCs) of healthy volunteers. The isolated $CD3^+$ T cells were stimulated with human CD3 and CD28 antibody-coated microbeads (α-CD3/28 beads) in the presence of interleukin-2 (IL-2). We then transduced the activated $CD3^+$ T cells with separate retroviral vectors that individually code for *OCT3/4*, *SOX2*, *KLF4*, and c-*MYC*. Human ESC-like colonies were obtained within 25 days of culture (Figure S1A available online).

We also isolated PBMCs from an HLA-A24-positive patient with a chronic HIV-1 infection. $CD8^+$ CTL clones specific for an antigenic peptide (amino acids [aa] 138–145) from the HIV-1 Nef protein (Nef-138-8(WT); RYPLTFGW) (Altfeld et al., 2006) were established. One of the clones, H25-#4, was stimulated using α-CD3/28 beads in the presence of IL-2 and then transduced simultaneously with six retroviral vectors encoding *OCT3/4*, *SOX2*, *KLF4*, c-*MYC*, *NANOG*, and *LIN28A*. However, we could not reprogram H25-#4 into pluripotency, possibly due to the cells being in a low infectious and exhausted state, or due to insufficient expression of the reprogramming factors. In response, we attempted to increase transduction efficiency and transgene expression by using two Sendai virus (SeV) vectors. One of them encodes tetracistronic factors (*OCT3/4*, *SOX2*, *KLF4*, and c-*MYC*) (Nishimura et al., 2011) with the miR-302 target sequence (SeVp[KOSM302L]; K.N., M.O., and M.N., data not shown), and another encodes SV40 large T antigen (SeV18[T]) (Fusaki et al., 2009). After transduction of phytohemagglutinin (PHA)-activated H25-#4 cells with the SeV vectors in the presence of IL-7 and IL-15, sufficient numbers of human ESC-like colonies appeared within 40 days of culture (Figure 1A). Use of this SeV system and optimization of transduction conditions greatly improved the reprogramming efficiency. It enabled us to reprogram several $CD8^+$ or $CD4^+$ T cell clones specific to pp65 antigen in cytomegalovirus (CMV), glutamic acid decarboxylase (GAD) antigen in type 1 diabetes, and α-GalCer (Table 1).

The resultant $CD3^+$ T cell- and H25-#4-derived ESC-like colonies (TkT3V1-7 and H254SeVT-3, respectively) exhibited alkaline phosphatase (AP) activity and expressed the pluripotent cell markers SSEA-4, Tra-1-60, and Tra-1-81 (Figures S1B–S1E and 1B–1E). H254SeVT-3 expressed HLA-A24 (Figure 1F). Both TkT3V1-7 and H254SeVT-3 also expressed human ESC-related genes (Figures S1F and 1G). The expression of exogenous reprogramming factors from the integrated provirus (TkT3V1-7) was halted (Figure S1F), and nonintegrated SeV genomic RNA was successfully removed from the cytosol by RNAi or by self-degradation caused by temperature-sensitive mutations (H254SeVT-3) (Figure 1H). Comparison of gene-expression profiles revealed that the gene-expression patterns in the ESC-like cells were similar to those in human ESCs, but differed significantly from those in peripheral blood (PB) T cells (Figure S1G). Scant methylation of the *OCT3/4* and *NANOG* promoter regions was confirmed using bisulfite PCR, thus indicating successful reprogramming (Freberg et al., 2007) (Figures S1H and 1I). In addition, when injected into nonobese diabetic severe combined immunodeficient (NOD-Scid) mice, those cells formed teratomas containing characteristic tissues derived from all three germ layers, which is indicative of pluripotency (Brivanlou et al., 2003) (Figures S3 and 2A). Therefore, those colonies were confirmed as typical human iPSCs.

T-iPSCs Carry Preassembled TCR Genes from the Original T Cell

Almost all TCRs are composed of heterodimerically associated α and β chains. *TCRA* or *TCRB* gene (encoding α chain or β chain, respectively) rearrangements are involved in normal αβ T cell development in the thymus. These rearrangements enabled us to determine retrospectively whether the iPSCs were derived from an αβ T cell. The BIOMED-2 consortium designed multiplex-PCR primers for analyzing *TCRB* gene assemblies (van Dongen et al., 2003), and we designed the primers for detecting *TCRA*

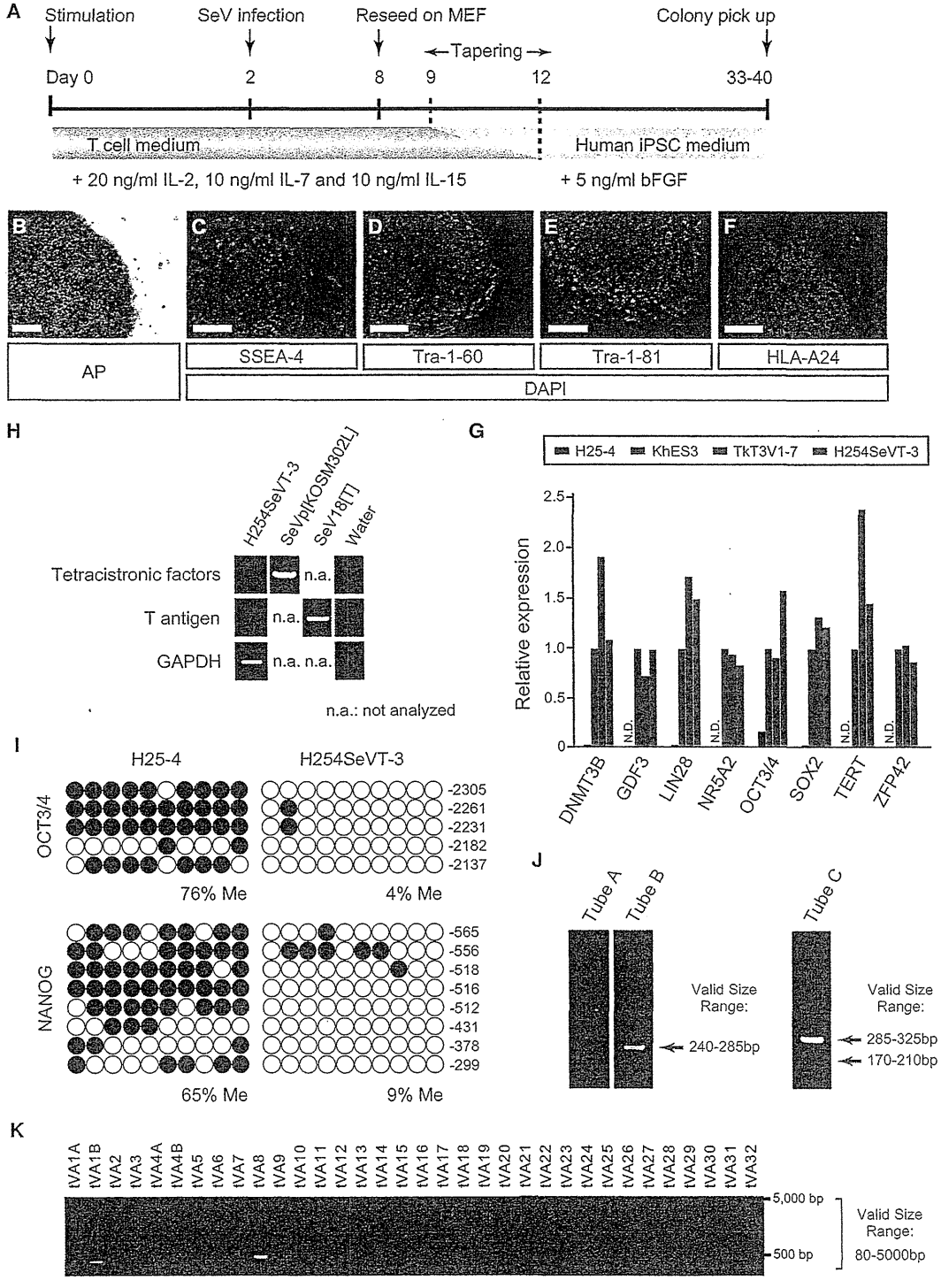

Figure 1. Generation of Human iPSCs from a CTL Clone

(A) Schematic illustration showing the generation of T-iPSCs from H25-#4 T cells using SeV vectors encoding polycistronic *OCT3/4*, *SOX2*, *KLF4*, and c-*MYC*, or SV40 large T antigen. The "tapering" indicates the gradual replacement of culture medium with human iPSC medium.

(B–F) AP activity (B) and expression of pluripotency markers (SSEA-4, C; Tra-1-60, D; and Tra-1-81, E) and HLA-A24 (F) in H254SeVT-3 cells. Nuclei were counterstained with DAPI. The scale bar represents 200 μm.

(G) Quantitative PCR for pluripotency genes in H25-#4, KhES3, TkT3V1-7, and H254SeVT-3 cells. Individual PCR reactions were normalized against 18S ribosomal RNA (rRNA).

(legend continued on next page)

Table 1. Generation of Human T-iPSCs from Various Patient-Derived T Cell Specimens

Antigen	T Cell Source	Initial Cell Number	No. of ESC-like Colonies	No. of Colonies Picked up for Establishing T-iPSC Clones	Date (MM/YYYY)
HIV-1 Nef	monoclonal T cell clone	4×10^5	7	7	05/2011
CMV pp65	polyclonal tetramer-sorted cells	~5,000	15	15	07/2011
GAD	monoclonal T cell clone	1×10^6	>100	not picked up	08/2012
		5×10^5	>100	19	08/2012
α-GalCer	FACS-sorted Vα24$^+$ cells	1×10^6	>100	not picked up	08/2012
		5×10^5	>100	7	08/2012

Sample cells were transduced with *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, and SV40 large T-antigen by using two Sendai virus (SeV) vectors (SeVp [KOSM302L] and SeV18[T]). After around 40 days, the number of embryonic stem cell (ESC)-like colonies were counted on the basis of morphology and alkaline phosphatase (AP) activity. All established T cell-derived induced pluripotent stem cell (T-iPSC) lines were free from residual SeV vectors (one example in the case of the HIV-1 Nef-specific T-iPSC clone is shown in Figure 1H). CMV, cytomegalovirus; GAD, glutamic acid decarboxylase; FACS, fluorescence-activated cell sorting.

gene assemblies (Figure S2). *TCRB* and *TCRA* gene assemblies were identified as single bands representing each allele in TkT3V1-7 and H254SeVT-3 (Figures S1H, S1I, 1J, and 1K).

We next confirmed the presence of an antigen-recognition site on the TCR that consisted of three complementarity-determining regions (CDR1, CDR2, and CDR3). CDR3 is the most diversifiable among the three because it spans the V(D)J-junction region, where several random nucleotides (N or P nucleotides) are inserted (Alt and Baltimore, 1982; Lafaille et al., 1989). We determined the CDR3 sequences of the assembled *TCRA* and *TCRB* genes in TkT3V1-7 and H254SeVT-3 and identified a set of productive *TCRA* and *TCRB* gene rearrangements (i.e., in-frame junction with no stop codon) (Table S1 and Table 2). Furthermore, the sequences of CDR3 from H254SeVT-3 and H25-#4 were completely identical at both *TCRA* and *TCRB* gene loci. These results indicated that the iPSCs established were derived from a single T cell and that the antigen specificity encoded in the genomic DNA of the T cell was conserved during reprogramming.

Redifferentiation of T-iPSCs into CD8 Single-Positive T Cells Expressing the Desired TCR

Following the application of specific in vitro differentiation protocols, iPSCs can give rise to mesoderm-derived cell types, especially hematopoietic stem and/or progenitor cells (Takayama et al., 2008; Vodyanik et al., 2005) (Figure 2B). This was applied to assess the capacity of T-iPSCs for hematopoietic differentiation by coculturing on C3H10T1/2 feeder cells in the presence of VEGF, SCF, and FLT-3L for the generation of CD34$^+$ hematopoietic stem and/or progenitor cells. On day 14 of culture, the cells were transferred onto Delta-like 1-expressing OP9 (OP9-DL1) feeder cells (Timmermans et al., 2009) and were cocultured in the presence of FLT-3L and IL-7 (Ikawa et al., 2010) (Figure 2B). After 21–28 days of culture, the hematopoietic cells differentiated into CD45$^+$, CD38$^+$, CD7$^+$, CD45RA$^+$, CD3$^+$, and TCRαβ$^+$ T lineage cells (Figure S4). As was the case with TCRαβ transgenic mice (Borgulya et al., 1992) and chimeric mice derived from ESCs produced through nuclear transplantation of T cells (Serwold et al., 2007), aberrant expression of TCRαβ was observed at the CD4/CD8 double-negative (DN) stage. Although some of these T lineage cells differentiated into the CD4/CD8 double-positive (DP) stage and the more mature CD4 or CD8 single-positive (SP) stages (Figure 2C), we could not characterize the small number of SP cells in more detail.

During thymocyte development, the CD4/CD8 DN and DP stages correspond respectively to the *TCRB*-encoded β chain and *TCRA*-encoded α chain assembly stages (von Boehmer, 2004). In the *TCRB* locus, the negative-feedback regulation of gene assembly and the capacity to deter further rearrangement are very strict (Khor and Sleckman, 2002). In the *TCRA* locus, by contrast, negative-feedback regulation is relatively loose, and further gene assembly of the preassembled gene, a phenomenon known as "receptor revision," tends to occur (Huang and Kanagawa, 2001; Krangel, 2009). In experiments using TCRα transgenic mice, the reactivation of *Rag1* and *Rag2*, genes related to recombination machinery, occurred in CD4/CD8 DP-stage thymocytes, and gene assembly of endogenous *Tcra* was observed (Padovan et al., 1993; Petrie et al., 1993). Such further gene assembly would be exceedingly undesirable for our purposes, because it would probably convert the tropism of the TCR and render the redifferentiated T cells incapable of attacking the previously targeted antigen. To determine whether such receptor revision could occur in redifferentiating T lineage cells, we collected CD1a$^-$ DN- and CD1a$^+$ DP-stage cells from among the CD45$^+$, CD3$^+$, TCRαβ$^+$, and CD5$^+$ T lineage cells and then analyzed the gene rearrangement of TCR messenger RNAs (mRNAs) (Figures S5A–S5C). Nucleotide sequences of *TCRB* mRNAs in the T lineage cells were identical to those in

(H) Detection of the remnants of SeV genomic RNAs by RT-PCR. Each column represents the template cDNA synthesized from H254SeVT-3 cells, SeVp [KOSM302L] virus solution, and SeV18[T] virus solution. cDNAs from virus solution were the positive controls.
(I) Bisulfite sequencing analyses of the *OCT3/4* and *NANOG* promoter regions in H25-#4 and H254SeVT-3 cells. White and black circles represent unmethylated and methylated (Me) CpG dinucleotides, respectively.
(J) Multiplex PCR analysis to detect *TCRB* gene rearrangements in the H254SeVT-3 genome. Tubes A and B contain Vβ-(D)Jβ assemblies; Tube C contains D-Jβ assemblies.
(K) Multiplex PCR analysis for detection of *TCRA* gene rearrangements (V-Jα assemblies).
See Figures S1, S2, and S3 for additional data.

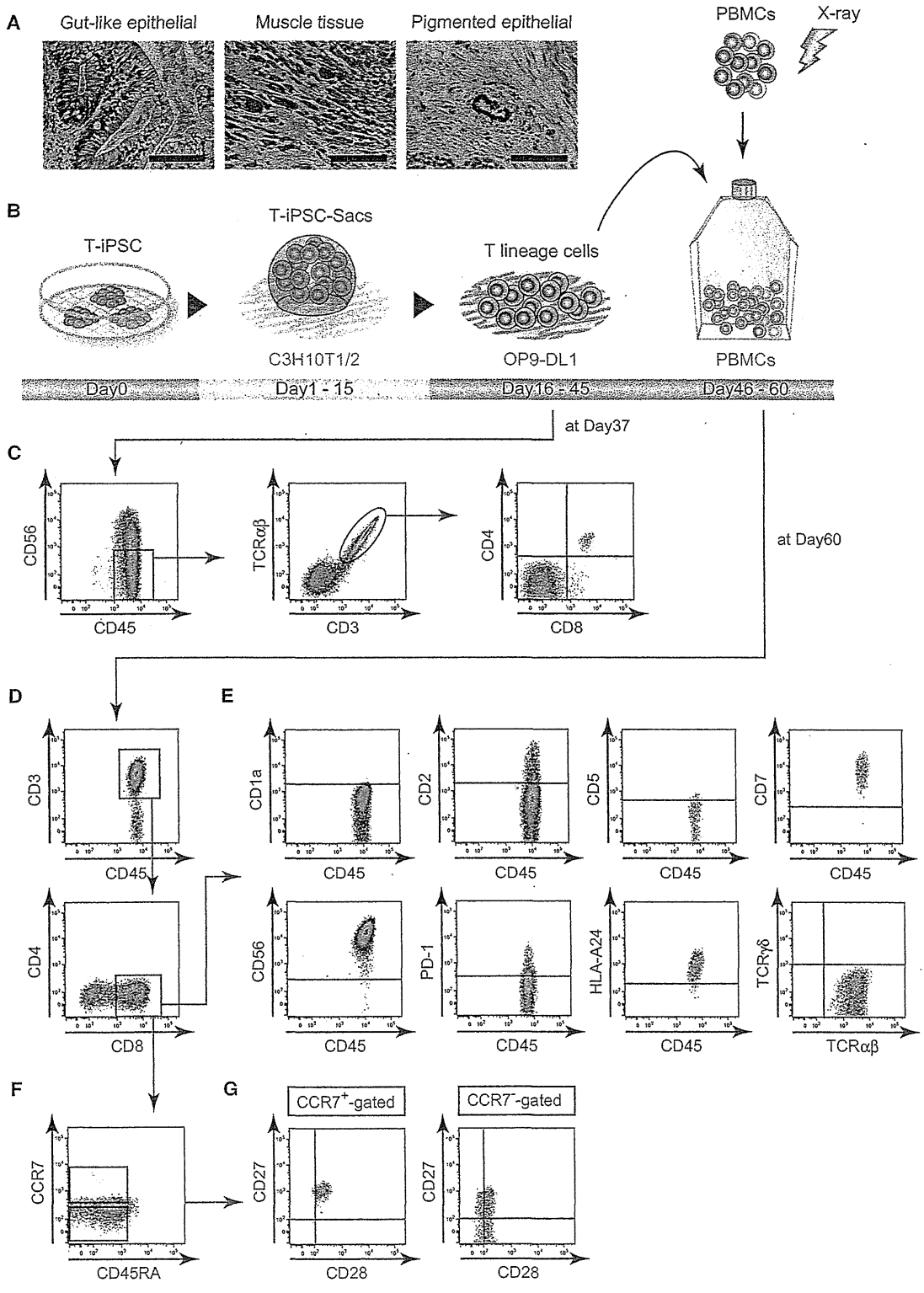

Figure 2. Redifferentiation of T-iPSCs into T Cells

(A) Representative hematoxylin- and eosin-stained sections of a teratoma formed in a NOD/ShiJic-*scid* mouse testis. H254SeVT-3 differentiated into cell lineages derived from endoderm (goblet cells in gut-like epithelial), mesoderm (smooth myocytes in muscle tissue), and ectoderm (retina cells in pigmented epithelial). The scale bar represents 100 μm.

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the T-iPSCs at both the DN and DP stages. By contrast, some *TCRA* mRNAs at the DN and DP stages were identical to those in the T lineage cells, but others differed, and differing sequences were observed more frequently at the DP stage than the DN stage (Table S2). *RAG1* and *RAG2* expression were observed at both the DN and the DP stages, though stronger expression was observed at the DP stage (Figure S5D).

To create mature CD8 SP cells from T-iPSC-derived T lineage cells without receptor revision, we focused on TCR signaling. Turka et al. (1991) reported that TCR signaling via peptide-major histocompatibility complex (MHC) complexes during positive selection ends expression of *RAG* genes and prevents further assembly of TCR genes. They also showed that mimicking TCR signaling using CD3 antibodies had the same effect. Therefore, we tried to stimulate the TCRs of redifferentiating T lineage cells before the completion of the DN-to-DP transition (Figure 2B). For this experiment, we cultured T lineage-committed cells on OP9-DL1, stimulated them with α-CD3/28 beads or PHA (we defined this as the first stimulation) and then cocultured them with irradiated HLA-A24$^-$ PMBCs in the presence of IL-7 and IL-15, which are required for the generation of memory phenotype CD8$^+$ T cells (Kaneko et al., 2009; Prlic et al., 2002; Tan et al., 2002). After 14 days, CD8 SP cells appeared (Figure 2D). These were deemed to be derivatives of H254SeV-3 based on their expression of HLA-A24 (Figure 2E). These CD8 SP cells did not express the immature thymocyte marker CD1a, but they were positive for CD56, which is expressed on CD8$^+$ T cells cultured in vitro (Lu and Negrin, 1994). In addition, these cells expressed CD7 and some CD2, but not CD5. On the one hand, they did not express PD-1, a marker of exhausted T cells (Figure 2E). On the other hand, some of them expressed the memory T cell markers CCR7, CD27, and CD28 simultaneously, thus representing a central memory T cell phenotype (Figures 2F and 2G) (Romero et al., 2007).

To test whether the redifferentiated CD8 SP cells would recognize the same epitope on the same HLA, the entire population of redifferentiated T cells was mixed with the A24/Nef-138-8(WT) tetramer and subjected to flow-cytometric analysis (Kawana-Tachikawa et al., 2002). Most of the CD8 SP cells were stained positively by the A24/Nef-138-8(WT) tetramer, but not by the control tetramer, which represents HIV-1 envelope-derived peptides (RYLRDQQLL; Figure 3A and data not shown). We then collected the A24/Nef-138-8(WT) tetramer-reactive CD8$^+$ cells and expanded them once again using α-CD3/28 beads or PHA stimulation (defined as the second stimulation; Figure 3A). Finally, after several independent redifferentiation experiments, we obtained A24/Nef-138-8(WT) tetramer-reactive CD8 SP cells (reT-1, reT-2.1, reT-2.2, and reT-3). As expected, sequence analysis of *TCRA* and *TCRB* mRNAs in the redifferentiated CD8 SP cells revealed that the TCR gene rearrangement pattern was identical to that in the H25-#4 original T cell clone (Figure 3B and Table 1).

To determine whether the redifferentiated CD8 SP cells were of the T cell lineage, we used quantitative PCR to compare gene-expression profiles among redifferentiated CD8 SP cells, PB CD4$^+$ and CD8$^+$ T cells, and the H25-#4 original T cell clone. As shown in Figure 3C, the expression patterns of CD3, CD4, and CD8 were similar among PB CD8$^+$ T cells, redifferentiated CD8 SP cells, and the H25-#4 original T cell clone. However, the pattern differed from those in PB CD4$^+$ T cells (Figure 3C). Cytotoxic "signature" genes such as granzyme B (*GZMB*), perforin (*PRF1*), interferon-γ (IFN-γ; *IFNG*), and FAS ligand (*FASLG*) were expressed in PB CD8$^+$ T cells. These genes were also expressed relatively strongly in redifferentiated CD8 SP cells and in the H25-#4 original T cell clone; that is, in already-primed T cells (Figure 3D). The expression patterns of several factors involved in transcription or signal transduction and of cell-surface molecules were similar among PB CD8$^+$ T cells, redifferentiated CD8 SP cells, and the H25-#4 original T cell clone (Figure 3E). To exclude the possibility that the redifferentiated CD8 SP cells had acquired natural killer (NK)-like properties during their coculture with OP9-DL1 or PBMCs, we used a complementary DNA (cDNA) microarray to analyze global gene-expression profiles in redifferentiated CD8 cells, the H25-#4 original T cell clone, and PB NK cells. Correlation and cluster analyses of the gene-expression profile of the redifferentiated CD8 SP cells showed it to be similar to that of the H25-#4 original T cell clone but different from that of NK cells (Figures 3F and 3G). These data strongly suggest that T-iPSCs are able to redifferentiate into CD8$^+$ T cells that exhibit the same antigen specificity as that of the original T cell.

Generation of Highly Proliferative T Cells through T-iPSCs

Fewer than 10^5 T lineage cells were obtained from ~3 × 10^5 T-iPSCs after coculture with C3H10T1/2 and OP9-DL1 cells. However, they could be expanded to >10^8 cells with the first stimulation (data not shown). After separating A24/Nef-138-8(WT) tetramer-reactive CD8$^+$ cells, we assessed the expansion rate induced by the second stimulation and also assessed the establishment of reT-1, reT-2.2, and reT-3. We found that these cells expanded from 100-fold to 1,000-fold within 2 weeks in the presence of IL-7 and IL-15, whereas the H25-#4 original T cell clone expanded only about 20-fold (Figure 4A). Even after 100-to-1,000-fold expansions, some cells still expressed central memory T cell markers such as CCR7, CD27, and CD28 (Figure S6). Perhaps with passage through the iPSC state, wherein telomerase activity is quite high (Marion et al., 2009; Takahashi et al., 2007), re-elongation of shortened telomeres in the H25-#4 original T cell clone gives the redifferentiated T cells high replicative potential (Monteiro et al., 1996; Weng et al., 1998). In fact, the redifferentiated T cells carried longer telomeres than the original T cell clone (Figure 4B), an overall process that we call

(B) Schematic illustration of redifferentiation from T-iPSCs into T cells.
(C) Flow-cytometric analysis of the phenotypes of differentiating T lineage cells at 37 days after starting redifferentiation.
(D and E) Flow-cytometric analysis of the phenotypes of T cells at 60 days after starting redifferentiation. Fluorescence-activated cell sorting (FACS) analyses revealed CD8 single-positive maturation (D) and expression of several T cell markers (E).
(F and G) Memory phenotypes of redifferentiated CD8$^+$ T cells. There existed memory-phenotyped cells such as all positive for CCR7 (F), CD27, and CD28 (G). Data are representative of at least three independent experiments. See Figures S3, S4, and S5 and Table S2 for additional data.

Table 2. TCR Gene Rearrangements in H25-4, H254SeVT-3, or Redifferentiated CD8⁺ T Cells

Cell	Genome or mRNA	Productivity	Rearrangement			Sequence of Junctional Region		
			Vα		Jα	3′Vα	P(N)	5′Jα
TCRA								
H25-4	genome	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
H254SeVT-3	genome	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
reT-1	mRNA	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
reT-2.1	mRNA	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
reT-3	mRNA	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
			Vβ	Dβ	Jβ	3′Vβ	N1-Dβ-N2	5′Jβ
TCRB								
H25-4	genome	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
		unproductive	germline	TRBD1*01	TRBJ2-7*01	TACAAAGCTGTAACATTGTG	GGGACAACT	CTACGAGCAGTACTTCGGGCCG
H254SeVT-3	genome	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
		unproductive	germline	TRBD1*01	TRBJ2-7*01	TACAAAGCTGTAACATTGTG	GGGACAACT	CTACGAGCAGTACTTCGGGCCG
reT-1	mRNA	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
reT-2.1	mRNA	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
reT-3	mRNA	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC

PCR-amplified samples (H25-4: not shown; H254SeVT-3: shown in Figures 1J and 1K; reT-1, reT-2.1, and reT-3: shown in Figure 3B) were sequenced, then V, D, and J segment usages and junctional sequences in CDR3 were identified. Following reprogramming and redifferentiation, there were no alterations in gene rearrangement in either allele at the *TCRA* and *TCRB* gene loci. See Table S1 for additional data on another T-iPSC clone (TkT3V1-7).

[a]Out-of-frame junction (at CDR3).

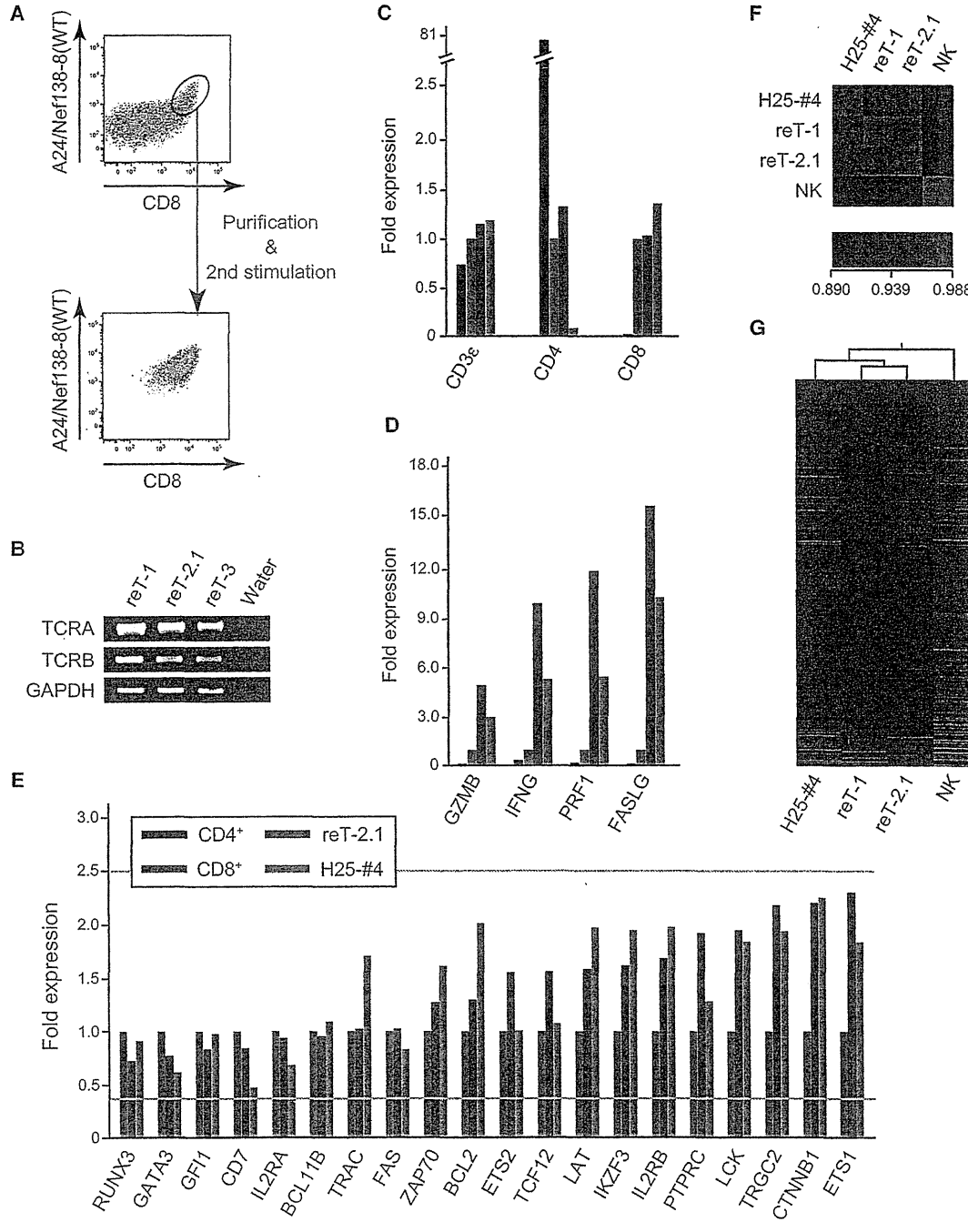

Figure 3. Characterizations of Redifferentiated T Cells as T Cells

(A) Recognition of A24/Nef-138-8(WT) tetramer at 50–60 days after starting redifferentiation, analyzed by flow cytometry (upper panel). Tetramer-positive cells were sorted by FACS or magnetically selected, then cultured for an additional 14 days, after which the expanded T cells were reanalyzed for tetramer (lower panel).

(B) TCR mRNAs were identified in a SMART-mediated cDNA library for reT-1, reT-2.1, and reT-3 cells. *GAPDH* is an internal control for PCRs.

(C–E) Quantitative PCR to compare the expression of major cell surface molecules (C), cell lytic molecules (D), and transcription factors and signal-transduction molecules (E) among PB CD4$^+$, PB CD8$^+$, reT-2.1, and H25-#4 cells. Individual PCR reactions were normalized against 18S rRNA.

(F and G) Global gene expression was analyzed using a cDNA microarray. Heat maps show the correlation coefficients between samples (F) and differential expression (>3-fold) of genes relative to NK cells (G). Red and green colorations indicate increased and decreased expression, respectively.

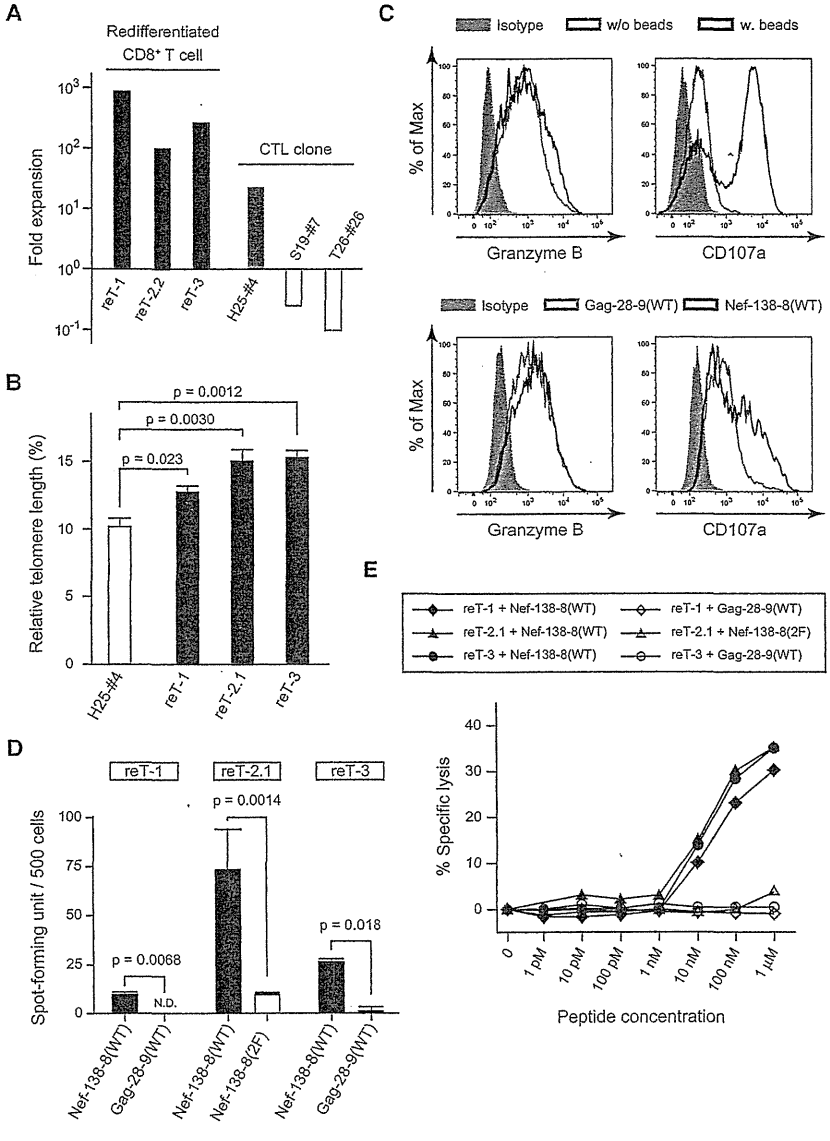

Figure 4. Redifferentiated T Cells Show T Cell Functionality and the Same Antigen Specificity as the Original CTL Clone

(A) Expansion ratios for reT-1, reT-2.2, and reT-3 cells elicited by PHA, IL-7, and IL-15 stimulation for 2 weeks. H25-#4 is the original clone. S19-#7 and T26-#26 were other Nef-138-8(WT)-specific CTL clones derived from different patients.

(B) Relative telomere length determined using flow-FISH. Data are presented as mean ± SEM.

(C) Intracellular production of granzyme B (left panel) and CD107a mobilization (right panel) induced by stimulation of reT-2.1 cells with α-CD3/CD28 beads or Nef-138-8(WT). Shaded plot: stimulated cells, isotype antibody; gray line: unstimulated cells, granzyme B or CD107a antibody; black line: stimulated cells, granzyme B or CD107a antibody.

(D) IFN-γ production in the presence of Nef-138-8(WT) measured using ELISPOT. Data are presented as mean ± SD. N.D., not determined.

(E) Standard ^{51}Cr release assay performed using the indicated concentrations of Nef-138-8(WT). Effector:target = 5:1.

See Figure S6 for additional data.

"rejuvenation." Throughout the experiments, neither autonomous cell expansion nor aberrant cell survival without cytokines as leukemia cells was observed (data not shown). Taken together, these data indicate that by passing through the T-iPSC state, cloned cytotoxic T cells can become "rejuvenated" to central memory-like T cells with excellent potential for proliferation and survival.

Redifferentiated CD8$^+$ T Cells Exhibit Antigen-Specific T Cell Functionality

To determine whether redifferentiated CD8$^+$ T cells exerted cytotoxic effects upon recognition of specific peptides in the context of an MHC, we performed functional assays using HLA-A24-positive B-LCL cells as antigen-presenting cells. Gag-28-9(WT) (KYKLKHIVW) is an antigenic peptide (aa 28–36) from the HIV-1 Gag protein (Altfeld et al., 2006), whereas Nef-138-8(2F) (RFPLTFGW) is a Tyr-to-Phe-substituted single-residue mutant form of Nef-138-8(WT). Both peptides were presented on HLA-A24 cells.

One of the major mechanisms by which CTLs induce cytotoxity is the secretion of cytolytic molecules triggered by TCR signaling. Intracellular staining revealed that the cytolytic molecule granzyme B was produced and stored in the granules of redifferentiated CD8$^+$ T cells (Figure 4C, left column). CD107a, also known as lysosomal-associated membrane protein 1 (LAMP1), is a granulocyte membrane protein that transiently appears at the cell surface and is coupled to degranulation (secretion of cytolytic molecules) of the stimulated CTLs, after which CD107a returns to the cytoplasm (Rubio et al., 2003). CD107a molecules on the cell surface were captured by a fluorochrome-conjugated antibody when redifferentiated CD8$^+$ T cells were stimulated with α-CD3/28 beads or Nef-138-8(WT) peptide, but not in the absence of the beads or Gag-28-9(WT) peptide (Figure 4C, right column). In the second experiment, we used the enzyme-linked immunosorbent spot (ELISPOT) assay to assess cytokine productivity per cell and confirmed that redifferentiated CD8$^+$ T cells produced significant levels of IFN-γ in response to stimulation by its specific antigen, Nef-138-8(WT) (Figure 4D). In a separate experiment, we used a ^{51}Cr release assay to investigate cytolytic capacity and found that redifferentiated CD8$^+$ T cells lysed ^{51}Cr-incorporated B-LCLs only when Nef-138-8(WT) was presented on B-LCLs (Figure 4E).

These results are highly indicative that redifferentiated CD8$^+$ T cells can release cytotoxic molecules and kill antigen-expressing target cells in an antigen-specific manner. Moreover,

monoclonal TCRs mediate highly precise cell targeting that should broaden the therapeutic window for antigen-specific T cell therapy by avoiding the troublesome mispairing TCRs that can occur with the commonly used exogenous TCR transfer technique for inducing antigen-specific T cells from hematopoietic stem cells or peripheral mature T cells (Bendle et al., 2010; Brenner and Okur, 2009).

DISCUSSION

Using a HIV-1-epitope-specific CTL clone as a model, we demonstrated here that the reprogramming into pluripotency of a T cell clone and the subsequent redifferentiation to mature functional $CD8^+$ T cells are possible. These redifferentiated $CD8^+$ T cells are highly proliferative naive cells with elongated telomeres, and they exert T cell functions in the same HIV-1-epitope-specific manner, permitting the inference that this process of reprogramming and redifferentiation can rejuvenate mature antigen-specific T cells.

Generation of iPSCs from T cells was initially difficult. On the basis of reports by Seki et al. (2010), we also found that SeV is suitable for the reprogramming of aged and exhausted fibroblasts, as well as of T cells. We also found that coexpression of SV40 large T antigen acted synergistically with the classic Yamanaka factors in enhancing the reprogramming efficiency of T cells. Therefore, SV40 large-T antigen introduction using the SeV vector system was also included in the protocol. Worth noting is that c-*MYC* is a known oncogene, and when it is inserted into the genomic DNA by the retroviral vector, it may become a risk for tumorigenesis in the generation of iPSCs. The same concern does not apply to SeV vector systems, given that the genomic RNA could be removed from the cytosol after reprogramming. Therefore, the utilization of SeV vectors both improved reprogramming efficiency and shielded redifferentiating cells from oncogene- or provirus-mediated tumorigenesis (Kohn et al., 2003).

In the redifferentiation experiments, mimicking TCR signaling led to CD8-linage specification without reassembly of *TCRA* genes. Preassembled TCR genes are a distinctive feature of T-iPSCs not found on other pluripotent stem cells. TCR$\alpha\beta$ is aberrantly expressed on redifferentiating CD4/CD8 DN cells, and the TCR signaling evoked results in the cessation of *RAG* expression. Serwold and colleagues reported that aberrantly early expression of TCR from preassembled *Tcra* and *Tcrb* following TCR signaling in murine thymocytes drives later lymphomagenesis (Serwold et al., 2010). They cautioned that T-iPSCs might confer risk for TCR-mediated lymphomagenesis. Therefore, the redifferentiation method will need to be further optimized and confirmed for clinical safety before application in practical treatments. This may be achieved by the use of an inducible suicide-gene system for eliminating unwanted tumors after injections (Hara et al., 2008; Veldwijk et al., 2004).

Immunological assays found that the redifferentiated $CD8^+$ T cells exerted T cell functions such as cytolytic activity, IFN-γ secretion, and degranulation in a normal manner when stimulated with their specific antigens. The most striking difference was in their proliferation capacity and elongated telomeres, which correlates with the central-memory T cell phenotype. Stem cell-like memory T cells (T_{SCM}) were recently identified as a subpopulation of T cells that has the capacity for self-renewal and that is multipotent and able to generate central memory, effector memory, and effector T cells (Gattinoni et al., 2011; Turtle et al., 2009). In a humanized mouse model, T_{SCM} cells reconstituted the T cell population more efficiently than other known memory subsets while mediating a superior antitumor response. It was found that inhibition of GSK3β enhances the generation of T_{SCM} in culture. Combining T-iPSC-mediated T cell rejuvenation with GSK3β inhibition may therefore enable efficient generation of T_{SCM} cells and permit highly effective immunotherapy along with the reconstitution of a normal T cell immune system.

Although these data suggest that rejuvenated T cells enjoy an advantage over the original T cell clone, it remains unclear whether these HIV-epitope-specific rejuvenated T cells are effective in improving the overall status of HIV infection. This is because the role of $CD8^+$ T cells in HIV infection appears to vary depending on the disease stage (Appay et al., 2000; Borrow et al., 1994; Brodie et al., 1999; Day et al., 2006; Koup et al., 1994). Evasion of the immune response through CTL escape is another important factor in HIV pathogenesis, and the escaped virus is a substantial hurdle for HIV therapies (Phillips et al., 1991). Therefore, this system may work best instead against tumors such as a melanoma, for which certain antigenic epitopes are known, or against viral infections other than HIV, for which the roles of $CD8^+$ cytotoxic T cells are more established. Nonetheless, the system described in our study will make it possible to preserve and to supply highly proliferative, functional $CD8^+$ T cells specific to a variety of HIV epitopes without worrying about exhaustion. It may also act as a valuable tool in better understanding the role of adoptive immunity in HIV infection.

Here, we have presented a proof of concept of $CD8^+$ T cell rejuvenation. The concept is not limited only to $CD8^+$ cytotoxic T cells. It may also be applied to $CD4^+$ helper or regulatory T cells to control desired or undesired immune reactions in the context of malignancies, chronic viral infections, autoimmune diseases, or transplantation-related immune disorders, if optimization of redifferentiation conditions can be achieved. Biological and technical challenges lie ahead, but the data presented in this work open new avenues toward antigen-specific T cell therapies that will supply unlimited numbers of rejuvenated T cells and will regenerate patients' immune systems.

EXPERIMENTAL PROCEDURES

Generation of Antigen-Specific CTL Clones

Nef138-8(WT)-specific CTL lines were induced from PBMCs of a patient chronically infected with HIV-1 who is positive for HLA-A24, as described (Kawana-Tachikawa et al., 2002). Each CTL line was expanded from a single-cell sorted tetramer$^+$ T cell, and the cells in every CTL line were confirmed for expression of only one kind of TCR$\alpha\beta$. For more details of CTL-clone establishment, see the Supplemental Experimental Procedures.

Generation of T-iPSCs

Human iPSCs were established from PB T cells or a CTL clone as described (Takayama et al., 2010), slightly modifying the culture conditions. In brief, T cells were stimulated by α-CD3/CD28 antibody-coated beads (Miltenyi Biotec) or by 5 μg/ml PHA-L (Sigma-Aldrich). The activated cells were transduced with reprogramming factors via retroviral or SeV vectors and were cultured in RH10 medium (RPMI-1640 supplemented with 10% human AB Serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin), which was

gradually replaced with human iPSC medium (Dulbecco's modified Eagle's medium/F12 FAM supplemented with 20% knockout serum replacer, 2 mM L-glutamine, 1% nonessential amino acids, 10 μM 2-mercaptoethanol, and 5 ng/ml basic fibroblast growth factor [bFGF]). The established iPSC clones were transfected with small interfering RNA L527 (Nishimura et al., 2011) using Lipofectamine RNAi Max (Invitrogen) for removal of SeV vectors from the cytoplasm.

Analysis of TCR Gene Rearrangement in Genomic DNA

Genomic DNA was extracted from approximately 5×10^6 cells using QIAamp DNA kits (QIAGEN) according to the manufacturer's instructions. For *TCRB* gene rearrangement analysis, PCR was performed according to BIOMED-2 protocols (van Dongen et al., 2003). For *TCRA* gene rearrangement analysis, PCR was performed using the primers shown in Figure S2 and LA Taq HS (TaKaRa). The PCR protocol entailed three amplification cycles (30 s at 95°C, 45 s at 68°C, and 6 min at 72°C); 15 amplification cycles (30 s at 95°C, 45 s at 62°C, and 6 min at 72°C); and 12 amplification cycles (15 s at 95°C, 30 s at 62°C, and 6 min at 72°C). The dominant band within the expected size range was purified using a QIAquick gel-extraction kit (QIAGEN) and was then sequenced. V, D, and J segment usages were identified by comparison to the ImMunoGeneTics (IMGT) database (http://www.imgt.org/) and by using an online tool (IMGT/V-QUEST) (Lefranc, 2003). Gene-segment nomenclature follows IMGT usage.

Analysis of TCR Gene Rearrangement in mRNA

A method based on the "switch mechanism at the 5′-end of the reverse transcript (SMART)" (Du et al., 2006) was used to synthesize double-stranded cDNAs (Super SMART cDNA synthesis kit; BD Clontech). Reverse transcription was conducted with the 3′ SMART CDS primer, SMART II A oligonucleotides (Super SMART cDNA synthesis kit), and PrimeScript Reverse Transcriptase (TaKaRa) for 90 min at 42°C. Double-stranded cDNA was then synthesized and was amplified with 5′ PCR Primer II A (Super SMART cDNA synthesis kit), and reagents were provided in an Advantage 2 PCR Kit (BD Clontech). The PCR protocol entailed 20 cycles of 5 s at 95°C, 5 s at 65°C, and 3 min at 68°C. The amplified double-stranded cDNA was used as templates in TCRA- or TCRB-specific amplification reactions. With forward primer (2^{nd}_5′-SMART) and reverse primer (3′-TRAC for *TCRA* or 3′-TRBC for *TCRB*), 25 cycles of amplification were performed (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C). PCR products were cloned into pGEM-T Easy Vector (Promega) and were sequenced.

T Cell Differentiation from T-iPSCs

To differentiate human iPSCs into hematopoietic cells, we slightly modified a previously described protocol (Takayama et al., 2008). Small clumps of iPSCs (<100 cells) were transferred onto irradiated C3H10T1/2 cells and cocultured in EB medium (Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum [FBS] and a cocktail of 10 μg/ml human insulin, 5.5 μg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine, 0.45 mM α-monothioglycerol, and 50 μg/ml ascorbic acid) in the presence of VEGF, SCF, and FLT-3L. Hematopoietic cells contained in iPSC sacs were collected and were transferred onto irradiated OP9-DL1 cells (provided by RIKEN BRC through the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology [MEXT]) (Watarai et al., 2010). The hematopoietic cells underwent T lineage differentiation on OP9-DL1 cells during coculture in OP9 medium (αMEM supplemented with 15% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin) in the presence of FLT-3L and IL-7. The T lineage cells were then harvested, mixed with irradiated HLA-A24$^-$ PBMCs, and cocultured in RH10 medium in the presence of IL-7 and IL-15.

Intracellular Staining

For intracellular staining of granzyme B, T cells were stimulated by α-CD3/28 beads or peptide-loaded HLA-A24$^+$ B-LCLs. After 2 hr, brefeldin A (5 μg/ml; Invitrogen) was added, with incubation for 4 hours more. Cells were then harvested and fixed in Fixation/Permeabilization solution (BD Biosciences). Intracellular staining was performed as per the manufacturer's protocol using Perm/Wash buffer (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated granzyme B antibody (BD Biosciences). For capturing CD107a transiently expressed on cell surfaces, T cells were incubated with α-CD3/28 beads or peptide-loaded HLA-A24$^+$ B-LCLs and were cultured with FITC-conjugated CD107a antibody (BioLegend) for 6 hr. Harvested cells were fixed and stained as described above. Data were acquired on FACSAria II equipment (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Measurement of Telomere Length by Flow-FISH

Telomere length was measured using a Telomere PNA Kit/FITC (DAKO) as previously described (Neuber et al., 2003).

ELISPOT and ^{51}Cr Release Assays

The antigen-specific responses of T cells were measured using an ELISPOT assay for IFN-γ and a standard ^{51}Cr release assay as described (Kawana-Tachikawa et al., 2002; Tsunetsugu-Yokota et al., 2003). HLA-A24$^+$ B-LCLs were used as antigen-presenting cells.

Statistics

All data are presented as mean ± SD. All statistics were performed using Excel (Microsoft) and Prism (GraphPad software) programs, applying two-tailed Student's t test. Values of $p < 0.05$ were considered significant. For additional details, see the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for microarray data reported in this paper is GSE43136.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.11.002.

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