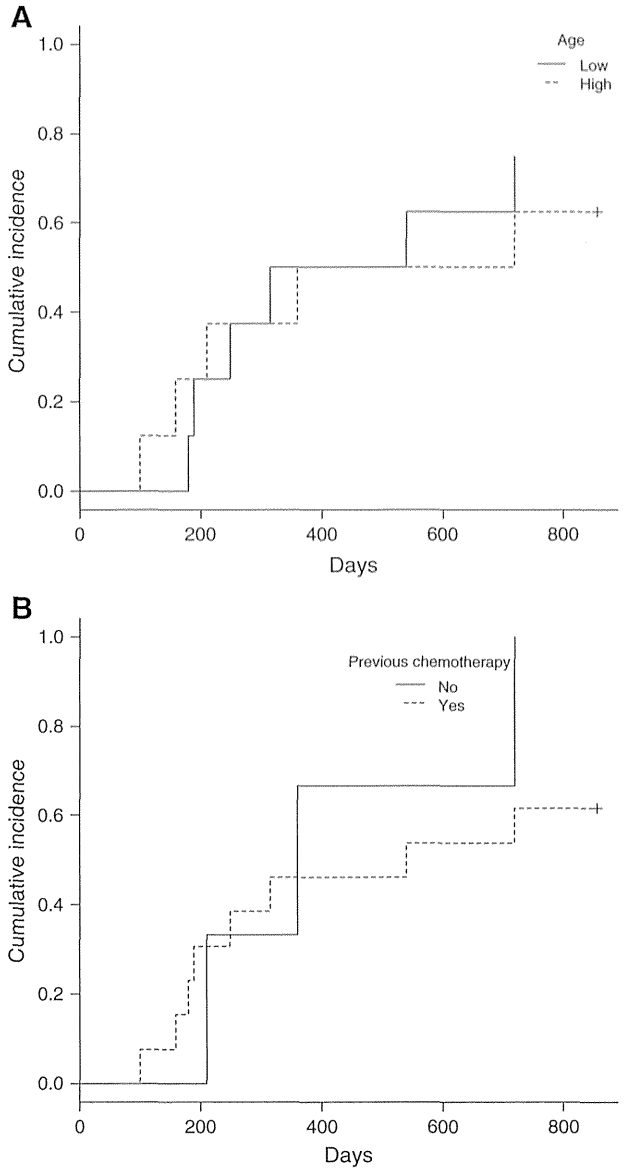

Fig. 3 Cumulative incidence of ovarian recovery grouped according to age (**a**) and the history of chemotherapy before transplantation (**b**)

the serum FSH is still higher than 100 mIU/mL at 2 years after HSCT. While it is unclear why only this patient has not achieved ovarian recovery, this may be due to the repeated intensive chemotherapies that were performed within a short duration due to the failure to achieve remission induction with the initial treatment. The effect of mitoxantrone on ovarian function compared to the other antineoplastic agents has not been clarified, but the dose intensity of this agent was higher in this patient (two courses of multiagent chemotherapy including mitoxantrone at 12 mg/m^2/day for 3 days). In fact, her serum FSH level before the conditioning regimen was already raised up to 107.73 mIU/mL.

Ovarian shielding was performed using different methods at the two institutions. However, there was no difference in the incidence of ovarian recovery. The facilities for TBI at SMC-JMU are widely available in many hospitals in Japan, and therefore, ovarian shielding can be performed in virtually all transplant centers. The incidence of ovarian recovery was similar between the younger patients and older patients, although the oldest patient in this study was 31 years old at HSCT. In addition, the effect of previous chemotherapy was not statistically significant. However, we should note that this study has poor statistical power due to the small sample size.

A major concern in performing ovarian shielding is the possibility of increasing the incidence of relapse of underlying hematological malignancies due to the decreased irradiation dose to the ovaries as well as other organs near the ovaries. However, the Seattle group reported that the incidence of relapse was not increased by the use of a non-myeloablative conditioning regimen that included only 2 Gy of TBI among patients in a complete remission of myelodysplastic syndrome or AML transformed from myelodysplastic syndrome (MDS) [12]. If we consider that the actual dose applied to the ovaries in these ovarian shielding protocols as measured by dosimeters was more than 2 Gy, the incidence of leukemia relapse may not increase if this procedure is performed in patients in remission. In this study, 4 of the total 16 patients developed relapse. Among these four patients, two each were in the first and second remission of acute leukemia at HSCT, respectively. The common characteristic of the two patients who developed relapse after HSCT in the first remission was a failure to achieve remission by the initial induction treatment. Furthermore, patients who underwent HSCT in remission received cyclosporine with a high target blood concentration [13]. This strategy strongly prevents GVHD at the possible expense of an increased relapse rate. In fact, there was only one non-relapse mortality in the total population. Thus, we do not consider that the incidence of relapse was increased by ovarian shielding. However, we should carefully monitor the incidence of relapse in patients who undergo TBI with ovarian shielding. In addition, we should provide patients with complete information, including the findings of the current study and the results of a previous decision analysis, when they have to decide whether or not to use ovarian shielding [14].

In conclusion, ovarian shielding by two distinct methods strongly protected ovarian function in young female patients undergoing HSCT. However, we should continue to monitor the relapse rate among patients who undergo this procedure. In addition, a certain amount of oocytes may be destroyed by TBI even with ovarian shielding, and therefore, recommendation for patients should be given concerning family planning based on the possibly reduced ovarian reserve. We are planning a prospective study of serial measurement of the anti-Müllerian hormone level as a marker for ovarian function in HSCT recipients.

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ORIGINAL ARTICLE

Single-cell T-cell receptor-β analysis of HLA-A*2402-restricted CMV- pp65-specific cytotoxic T-cells in allogeneic hematopoietic SCT

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Cellular immunity is important for the control of CMV infection after allogeneic hematopoietic cell transplantation (Allo-HCT). However, the actual *in vivo* dynamics of CMV-specific cytotoxic T cell (CMV-CTL) clones are still unclear. We conducted clone monitoring of tetramer$^+$ CMV-CTLs in HLA-A*2402-positive donor–patient pairs, using a direct single-cell analysis that enabled the simultaneous identification and quantification of CTL clones. Clone dynamics were assessed in three cases with or without CMV reactivation. In Case-1 without CMV reactivation, despite the long-term use of systemic steroid, dominant clones of Donor-1 persisted and remained dominant. The CMV-CTLs at 1 year after Allo-HCT included a high proportion of CD45RA$^+$CCR7$^-$ effector and CD27$^-$CD57$^+$ mature T cells. On the other hand, in Cases-2 and -3 with CMV reactivation, novel clones appeared and became dominant during the follow-up. Their CMV-CTLs included more CD27$^+$ immature T cells at 1 year after Allo-HCT. With regard to clonotypes, HLA-A*2402-restricted CMV-CTLs tended to select BV7 and BJ1-1 genes for complementarity-determining region 3 (CDR3) of T-cell receptor (TCR)-β. Specific amino-acid sequences of CDR3 of TCR-β were found in each case. Patterns of clone reconstitution and phenotype would be different according to CMV reactivation. *In vivo* clone monitoring of CMV-CTLs could provide insight into the mechanism of immunological reconstitution following Allo-HCT.

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Keywords: HLA-A*2402-restricted CMV-specific cytotoxic T cells; T-cell receptor-β; single-cell analysis; clone monitoring

INTRODUCTION

CMV reactivation is one of the major concerns after allogeneic hematopoietic cell transplantation (Allo-HCT).[1,2] It is important to control CMV reactivation before it progresses to diseases such as pneumonia because CMV diseases are associated with high mortality.[3,4] In addition to recent pre-emptive therapy, immunological reconstitution against CMV is necessary for long-term suppression of CMV reactivation.

Cellular immunity by CMV-specific cytotoxic T cells (CMV-CTL) is considered to have a major role in the control of CMV reactivation after Allo-HCT. HLA-restricted CMV-CTLs are identified by tetramer methods.[5,6] An individual CTL has a specific complementarity-determining region 3 (CDR3) of the T-cell receptor (TCR)-β, which is a result of the recombination of somatic TCR V-(D)-J genes and junction diversity. The repertoire of TCR and clones of CMV-CTLs have thus far been identified after *in vitro* bulk expansion, and therefore the results obtained to date may have been affected by the potential proliferation of CMV-CTL clones and bacterial-transforming efficiency.[7–10] Recently, we and others have developed a direct single-cell RT-PCR analysis that enables the simultaneous identification and quantification of CTL clones without the effects of *in vitro* expansion.[11,12] To the best of our knowledge, the *in vivo* dynamics and monitoring of CMV-CTL clones have not been assessed in Allo-HCT. In addition, the clonotypes in HLA-A*2402-restricted CMV-CTLs have not been clarified, even though HLA-A*2402 is the most common HLA-A allele in the Japanese population (~60%).

In this study, we investigated the TCR-β repertoire and clone monitoring of HLA-A*2402-restricted CMV-pp65$_{341–349}$ (QYDP-VAALF)-specific CTLs (CMV-pp65 CTLs) in Allo-HCT and found three distinct patterns of reconstitution for CMV-pp65 CTL clones.

PATIENTS AND METHODS

Patients and cells

This study included three pairs of recipients and their respective related donors. All of the recipients and donors were CMV-seropositive and had HLA-A*2402. The recipients received myeloablative conditioning using CY and TBI, and thereafter PBSC transplantation on day 0. Prophylaxis of GVHD was performed with CYA and short-term MTX. CMV reactivation was monitored weekly using a CMV antigenemia assay by the C10/11 method.

Peripheral blood samples were obtained from donors during the mobilization by G-CSF, whereas those from recipients were obtained every 1–3 months after Allo-HCT. Mononuclear cells were separated by density gradient sedimentation using Lymphoprep (Axis-Shield PoC AS, Dundee, Scotland) and were cryopreserved at −80 °C until use.

This study was approved by the institutional review board of Jichi Medical University and all subjects gave their written informed consent for the cryopreservation and analysis of the blood samples in accordance with the Helsinki declaration.

Staining and monitoring of HLA-A*2402-restricted CMV-pp65 CTLs

Cells were incubated with HLA-A*2402 CMV-pp65$_{341–349}$ peptide (QYDP-VAALF)-binding HLA tetramer (CMV tetramer) (Medical & Biological Laboratories, Nagoya, Japan), and then stained with anti-human CD3,

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CD8, CD4, CD14, CD16, CD19 and CD56 mAb (BD Biosciences, Tokyo, Japan). Data were analyzed with FACSCalibur and CellQuest (BD Biosciences). HLA-A*2402-restricted CMV-pp65 CTLs were defined as $CD8^+$CMV-tetramer$^+$ T cells. The proportions of CMV-pp65 CTLs to whole $CD8^+$ T cells were monitored every 1–3 months. We described the sampling phases as follows: the early phase, 1–3 months after Allo-HCT; the intermediate phase, about 6–9 months and the late phase, over 1 year after Allo-HCT.

Furthermore, phenotypic analysis was performed using non-cultured samples when sufficient CMV tetramer$^+$ T cells were detected for the first time after Allo-HCT and thereafter every 4–6 months: (Case-1) days 40, 215 and 376; (Case-2) days 58, 240 and 388; (Case-3) days 92, 264 and 416. Cells were incubated with CMV tetramer, and then stained with anti-human CD3, CD8, CD45RA and CCR7 mAbs (BD Biosciences). T cells were divided into four differentiation subsets according to their phenotypes: $CD45RA^+CCR7^+$ naive T cell, $CD45RA^-CCR7^+$ central memory T (T_{CM}) cells, $CD45RA^-CCR7^-$effector-memory T (T_{EM}) cells and $CD45RA^+CCR7^-$effector T (T_{EF}) cells.[6,13–15] Additionally, late-phase $CD8^+$CMV-tetramer$^+$ cells were also stained with CD27 and CD57 mAB (BD Biosciences). The maturation of T cells was defined according to the expression of CD27 and CD57:[16,17] $CD27^+CD57^-$immature T cells, $CD27^+CD57^+$, $CD27^-CD57^-$ T cells and $CD27^-CD57^+$mature T cells. Data were analyzed using FACSAria II and Diva software (BD Biosciences). The median absolute numbers of accepted CMV-tetramer-positive cells for phenotypic analysis were 639 cells (range:118–2157) in Case-1, 93 cells (range: 61–122) in Case-2 and 80 cells (range: 41–153) in Case-3, respectively (Supplementary Figure).

Single-cell TCR-β analysis of individual HLA-A*2402-restricted CMV-pp65 CTLs and clone monitoring

Clone identification by single-cell analysis was performed. The clone dynamics of each recipient was assessed using the same samples for phenotypic analysis. In Case-3, the proportions of CMV-tetramer$^+$ T cells were extremely low, and therefore samples on days 264 and 416 were combined with those on days 290 and 451, respectively. Thereafter, individual $CD3^+CD8^+$CMV-tetramer$^+$T cells were directly sorted as single cells into PCR tubes or microplates using FACSAria II (BD Biosciences). A median of 120 cells was sorted (range: 73–165) for each sample. The amino-acid (AA) sequences in CDR3 of TCR-β for sorted cells at a single-cell level were directly analyzed and determined after reverse transcript (RT)-PCR for TCR-β gene amplification as described previously.[11,12] In brief, individual CMV-pp65 tetramer$^+$ T cells were sorted at a single-cell level into PCR tubes. After direct cell lyses, cDNAs of TCR-β were synthesized by RT, with a TCR-β constant region gene-specific primer. The synthesized cDNA of TCR-β were used for two sequential steps of semi-nested PCR using 24 kinds of TCR-β variable region (BV) gene family-specific primers and two kinds of TCR-β constant primers. After we identified the BV family of individual cells, we directly sequenced AA of V-D-J CDR3 of T cells. The average efficiency of TCR analysis was 68: 77 in Case-1, 70 in Case-2 and 58% in Case-3.

Induction of CMV-pp65 CTLs in donor cells by culture in bulk

To compare the differences in the distribution of CMV-CTL clones between cultured and non-cultured cells, we cultured donor cells with 10 μg/mL of CMV-pp65$_{341–349}$ peptide (QYDPVAALF) (Sigma Genosys, Tokyo, Japan) in RPMI 1640 (Sigma, Tokyo, Japan) containing 10% fetal bovine serum (FBS). One day after peptide stimulation, 50 IU/mL of IL-2 (Shionogi, Osaka, Japan) were added to the medium. Thereafter, the medium was replaced with fresh medium that contained the same concentration of IL-2, as determined by the medium color. After 2 weeks, the cultured cells were collected and the repertoire of TCR-β in CMV-pp65 CTLs was analyzed as described above.

Establishment of a CMV-pp65 CTL clone from a single cell

To assess the cytotoxic effect of CMV-pp65 CTLs, CTL clones were established from the sample from Case-1 at 334 days after Allo-HCT. First, cells were cultured with 10 ng/mL of CMV-pp65$_{341–349}$ peptide in AIM-V (Life Technologies, Tokyo, Japan) with 10% human AB serum (Sigma). Two days after peptide stimulation, 50 IU/mL of IL-2 (Shionogi) were added. Thereafter, the medium was replaced with fresh medium that contained the same concentration of IL-2, as determined by the medium color. After 2 weeks, the cultured cells were collected and stained with CMV tetramer, anti-human CD3, CD8 and 7AAD mAbs (BD Biosciences). The $CD8^+$CMV-tetramer$^+$ T cells were sorted at a single-cell level into 96-well microplates with 100 μL/well of AIM-V containing 10% human AB serum, 5 μg/mL of PHA, 200 IU/mL of IL-2, and allo-feeder cells treated by mitomycin C. After 1 week, an additional 100 μL/well of AIM-V containing 10% human AB serum and 200 IU/mL of IL-2 were suspended in each well. After 4–6 weeks, cells were collected and we determined whether or not the proliferated cells were $CD8^+$CMV-tetramer$^+$ T cells. In Cases-2 and -3, CMV-pp65 CTL clones did not proliferate sufficiently.

Cytotoxicity of CMV-CTL clones

A fluorochromasia cytotoxicity assay was performed with a TERASCAN VPC system (Minerva Tech, Tokyo, Japan) according to previous reports.[18–20] Briefly, T2–24 cells that expressed HLA-A*2402 (kindly provided by Professor Kawakami of Keio University) were pulsed with 10 μg/mL of CMV-pp65$_{341–349}$ peptide (QYDPVAALF) or 10 μg/mL of HIV env gp 160 (RYLRDQQLL) (Sigma Genosys) for 1 h. The T2–24 cells as a target were then stained with calcein AM (WAKO, Osaka, Japan) for 30 min. After being washed three times, 1×10^4 T2–24 cells per well were suspended in a 96-well half area plate (Corning, Tokyo, Japan) and cultured with individual CMV-pp65 CTL clones in a dose-dependent manner. After 4 h, the release of calcein was measured and cytotoxicity was calculated.

RESULTS

Proportions and phenotypes of HLA-A*2402-restricted CMV-pp65 CTLs during the clinical courses

The patient backgrounds are shown in Table 1. Engraftment was successfully achieved in all three cases. Case-1 did not have CMV reactivation (Figure 1a). Case-1 experienced grade 2 acute GVHD and extensive chronic GVHD at 40 and 314 days after HCT, respectively. Although both the acute and chronic GVHD required steroid treatment, clinical CMV reactivation did not occur during the follow-up. Donor-1 showed a 0.66% CMV-pp65 CTLs, which included 67% $CD45RA^+CCR7^-T_{EF}$ and 27% $CD45RA^-CCR7^-$ T_{EM} cells (Figure 1b). The CMV-pp65 CTLs in the recipient appeared at 40 days after HCT and accounted for 5.2% of $CD8^+$ T cells. During the first 3 months, CMV-pp65 CTLs maintained a proportion of about 5% of $CD8^+$ T cells. Thereafter, the proportion of CMV-pp65 CTLs decreased, but remained at about 1–2% (Figure 1a). The proportion of $CD45RA^+CCR7^-T_{EF}$ cells in CMV-pp65 CTLs remained at 60–80% after Allo-HCT (Figure 1b).

Case-2 showed a CMV reactivation only in the early phase (Figure 1c). Case-2 did not experience acute GVHD, but developed

Table 1. Patient background

	Age		*Gender*		*Disease*	*CMV status*		*Conditioning*	*GVHD prophylaxis*	*CMV reactivation*
	Recipient	*Donor*	*Recipient*	*Donor*		*Recipient*	*Donor*			
Case-1	16	14	Male	Male	AML	+	+	CY-TBI	CsA + MTX	None
Case-2	36	33	Male	Male	AML	+	+	CY-TBI	CsA + MTX	Only in the early phase
Case-3	21	49	Male	Female	Acute promyelocytic leukemia	+	+	CY-TBI	CsA + MTX	Frequently

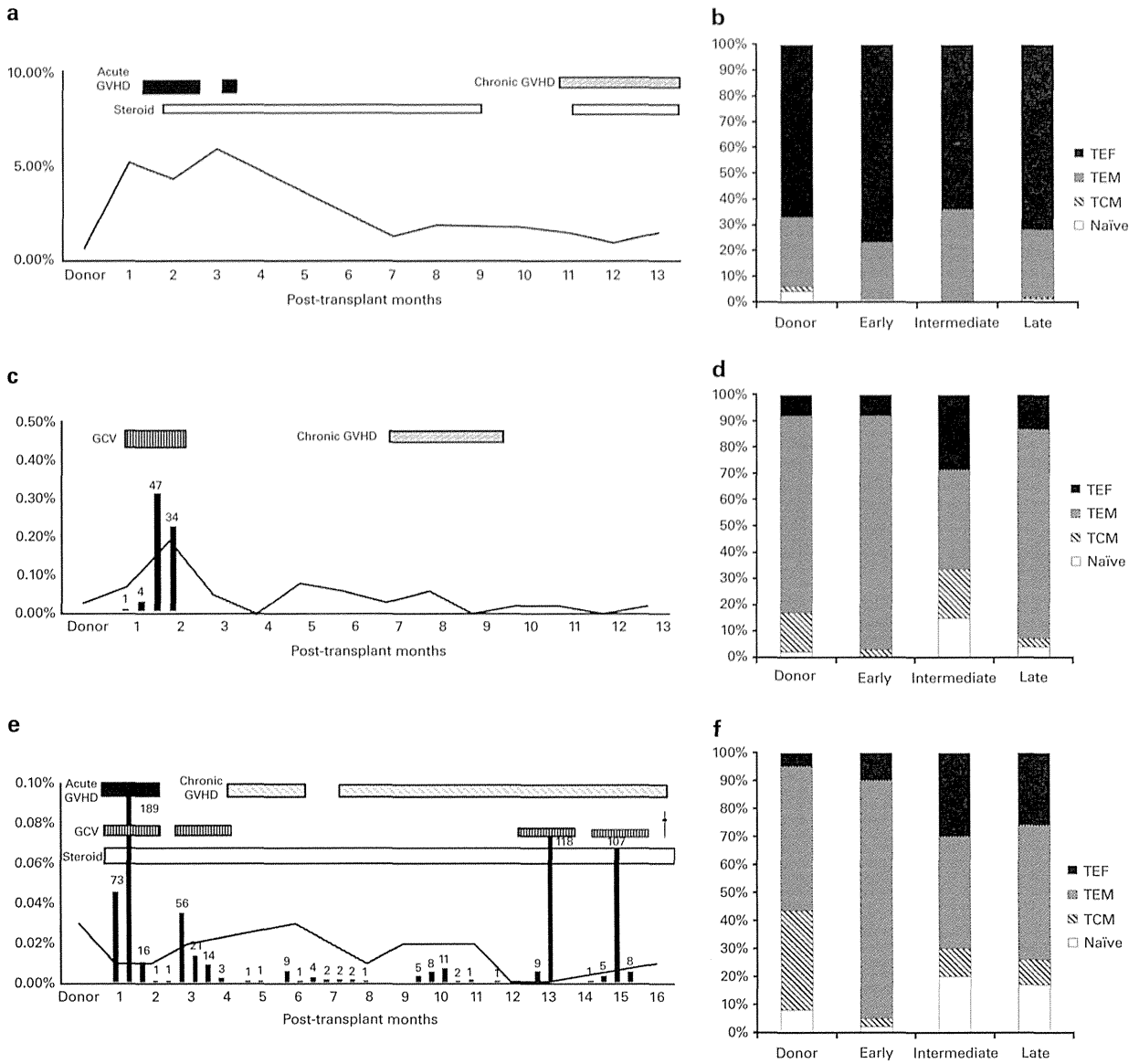

Figure 1. Clinical events and phenotypic dynamics of HLA-A*2402-restricted CMV-pp65-specific CTL (CMV-pp65 CTLs) in (**a** and **b**) Case-1, (**c** and **d**) Case-2 and (**e** and **f**) Case-3. (**a, c** and **e**) Clinical events and monitoring of the proportions of CMV-pp65 CTLs to whole CD8+ T cells; black vertical bars indicate CMV reactivation shown with counts of CMV antigenemia by the C10/11 method. (**b**, **d** and **f**) The dynamics of subpopulations of CMV-pp65 CTLs: CD45RA+CCR7+ naive T cells, CD45RA-CCR7+ central memory T cells (TCM), CD45RA-CCR7-effector-memory T cells (TEM) and CD45RA+CCR7-effector T cells (TEF). Early phase: days 40, 58 and 92; intermediate phase: days 215, 240 and 264; and late phase: days 376, 388 and 416 in Cases-1, -2 and -3, respectively.

limited chronic GVHD, which did not require steroid treatment. Donor-2 had 0.03% CMV-pp65 CTLs, almost all of which were $CD45RA^-CCR7^-$ T_{EM} cells (Figure 1d). Clinical CMV reactivation occurred 35 days after Allo-HCT and disappeared with GCV treatment. The CMV-pp65 CTLs accounted for 0.19% of $CD8^+$ T cells at 2 months after HCT. Thereafter, the proportion of CMV-pp65 CTLs decreased and remained at about 0.02–0.08% (Figure 1c). The CMV-pp65 CTLs after Allo-HCT included $CD45RA^+CCR7^-$ T_{EF} cells, but most of the CMV-pp65 CTLs were $CD45RA^-CCR7^-$ T_{EM} cells (Figure 1d).

Case-3 showed repeated CMV reactivation and experienced grade 4 acute GVHD and extensive chronic GVHD. He required long-term systemic steroid treatment (Figure 1e). Donor-3 had 0.03% CMV-pp65 CTLs in $CD8^+$ T cells. The CMV-pp65 CTLs included 52% $CD45RA^-CCR7^-T_{EM}$ and 36% $CD45RA^-CCR7^+$ T_{CM} cells, but few $CD45RA^+CCR7^-T_{EF}$ cells (Figure 1f). During follow-up, CMV-pp65 CTLs did not exceed 0.03% of $CD8^+$T cells (Figure 1e). The CMV-pp65 CTLs detected after Allo-HCT always included less $CD45RA^+CCR7^-T_{EF}$ cells than $CD45RA^-CCR7^-$ T_{EM} cells (Figure 1f).

Clonotypes of non-cultured HLA-A*2402-restricted CMV-pp65 CTLs

The clonotypes of CMV-pp65 CTLs were assessed in a total of 993 cells: 330 cells in Case-1, 335 cells in Case-2 and 268 cells in Case-3. BV7 were used among the BV family in 21, 16 and 35% of CMV-pp65 CTLs in Cases-1, -2 and -3, respectively. In addition, BJ1-1 was used among the BJ family in 21, 35 and 26% of CMV-pp65 CTLs in Cases-1, -2 and -3, respectively (Figure 2).

Of the total 993 cells, 187 clones were identified: 30, 74 and 83 in Cases-1, -2 and -3, respectively. Table 2 shows the features of clones that appeared in three or more cells during the follow-up. Within each case, specific AA sequences of CDR3 were found: DPG in Case-1, GQG and PRD in Case-2, and QVS in Case-3. Although no common AA motif of CDR3 was found among the three cases, GGGG was seen in the dominant clones in both Cases-1 and -3 (Table 2).

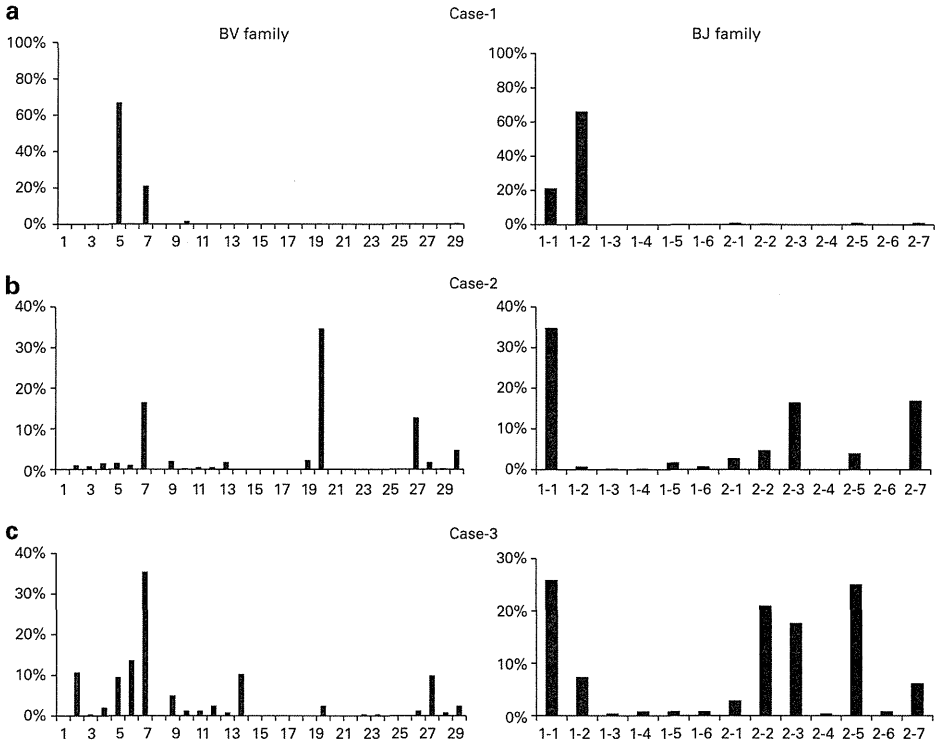

Figure 2. BV and BJ gene usages in HLA-A*2402-restricted CMV-pp65-specific cytotoxic T cells observed in (**a**) Case-1, (**b**) Case-2 and (**c**) Case-3.

Table 2. Clonotypes of CDR3 of TCR-β in HLA-A*2402-restricted CMV-specific CTLs, which appeared in three or more cells during follow-up

Case	*Clone-ID*	*Number of cells*	*BV*	*Amino acid of CDR3*	*BJ*
Case-1 (330 cells)	N-1	231	BV5	CASS**DPG**DSLNGYTF	BJ1-1
	N-2	66	BV7	CASS*STGGGG*TEAFF	BJ1-1
	N-9	6	BV10	CAIS**DPG**HRFGEAFF	BJ1-1
Case-2 (335 cells)	S-1	128	BV20	CSARFS**GQG**TEAFF	BJ1-1
	S-2	50	BV7	CASSSNQGATQYF	BJ2-3
	S-3	7	BV13	CASINRGTDPYEQYF	BJ2-7
	S-4	42	BV27	CAST**PRD**RSNYEQYF	BJ2-7
	S-7	9	BV7	CASSFTLSPETQYF	BJ2-5
	S-24	3	BV4	CASSQ**GQG**AQNQPQHF	BJ1-5
	S-32	18	BV30	CAGTGIRSAGELFF	BJ2-2
	S-56	4	BV28	CASK**PRD**SGDTQYF	BJ2-3
	S-57	3	BV20	CSARNPGLYEQYF	BJ2-7
Case-3 (268 cells)	I-1	18	BV7	CASSSIQGAAGELFF	BJ2-2
	I-2	24	BV14	CASS**QVS**TGETQYF	BJ2-5
	I-3	20	BV2	CASRG**QVS**SQETQYF	BJ2-5
	I-4	3	BV2	CAIKGTVSTQETQYF	BJ2-5
	I-24	50	BV7	CASSST*GGGG*AEAFF	BJ1-1
	I-25	25	BV6	CASDRGGNTGELFF	BJ2-2
	I-26	8	BV5	CASSLDTDTQYF	BJ2-3
	I-27	10	BV28	CASSPDRGRGYTF	BJ1-2
	I-28	7	BV7	CASSLSSTRTDTQYF	BJ2-3
	I-29	7	BV28	CASSLNSVGTEAFF	BJ1-1
	I-30	3	BV28	CASSSDNAIGGGSYGYTF	BJ1-2
	I-32	8	BV5	CASSLGAGGPSDTQYF	BJ2-3
	I-34	3	BV7	CASSLGGLAPSTDTQYF	BJ2-3

Abbreviations: BV = TCR-β variable region; CDR3 = complementarity-determining region 3; TCR = T-cell receptor. Bold letters indicate shared motifs of CDR3 within each case. Italic letters indicate a shared motif of CDR3 between Case-1 and Case-3.

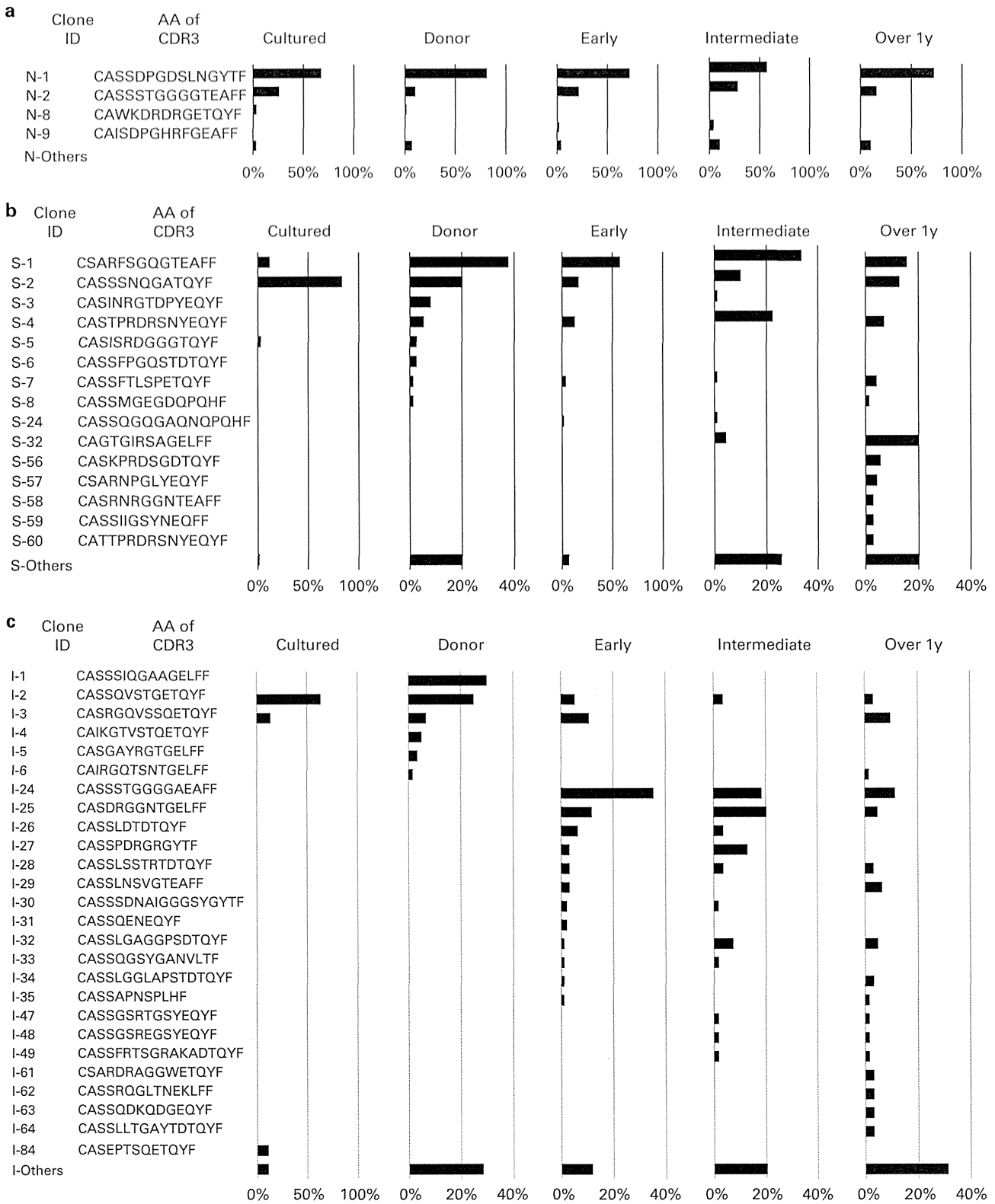

Figure 3. Clone monitoring of HLA-A*2402-restricted CMV-pp65-specific cytotoxic T cells in donors and recipients in the early, intermediate and late phases. Clones, which appeared at two or more points/two or more clones at one point, are shown with the clone-ID and amino acid (AA) sequences of CDR3 of T-cell receptor-β in (**a**) Case-1, (**b**) Case-2 and (**c**) Case-3. In addition, clones of cultured donor cells were also shown. Early phases: days 40, 58 and 92; intermediate phase: days 215, 240 and 264 and 290; and late phase: days 376, 388 and 416 and 451 in Cases-1, -2 and -3, respectively.

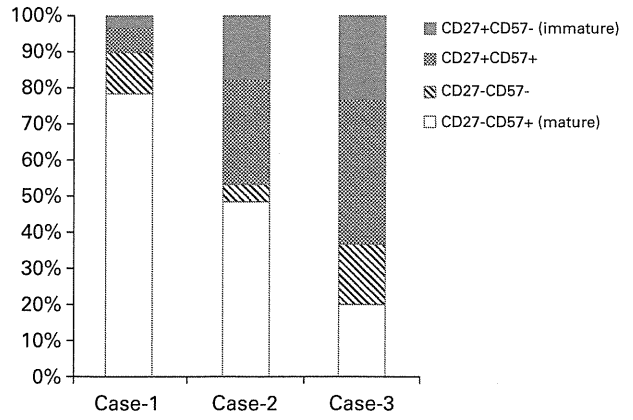

Figure 4. Maturation of HLA-A*2402-restricted CMV-pp65 CTLs among cases.

Clone monitoring of HLA-A*2402-restricted CMV-pp65 CTLs

Serial clone monitoring was performed using non-cultured donor cells, and recipient cells of early, intermediate and late phases (Figures 3a–c). In Case-1, who did not show CMV reactivation during the follow-up, the dominant CMV-pp65 CTL clones in the donor were well preserved in the recipient after Allo-HCT (Figure 3a). Especially, clone N-1 with CAISDPGHRFGEAFF of CDR3-AA always exceeded 50% in Case-1. Few novel clones (<10%) appeared during the follow-up.

In Case-2, who developed CMV reactivation only within the early phase after HCT, the dominant CMV-CTL clones in the donor were preserved in the early phase after Allo-HCT. However, novel clones appeared and gradually became dominant in a later phase (Figure 3b). At any point, at least one clone exceeded 20%.

In Case-3, who showed repeated CMV reactivation, some of the donor-dominant CMV-CTL clones were preserved in the early phase, but the novel clone I-24 with CASSSTGGGGAEAFF was the most dominant in the early phase (Figure 3c). However, the clones became more diverse during the follow-up, and eventually none of the clones exceeded 20% in the later phase.

The maturation analysis was also performed using samples of the later phase. $CD27^-CD57^+$ mature T cells accounted for 80% of the CMV-pp65 CTLs in Case-1, in whom donor-dominant clones remained dominant. On the other hand, the amount of $CD27^+$ immature T cells was increased in Cases-2 and -3, both of whom showed novel clones (Figure 4).

Difference in HLA-A*2402-restricted CMV-pp65 CTL clones between cultured and non-cultured cells in respective donors

CMV-pp65 CTL clones were compared between cultured and non-cultured cells of the donors. In Case-1, the dominant clones in cultured cells were detected in non-cultured cells and the proportions of dominant clones were similar (Figure 3a). However, in both Cases-2 and -3, the proportions of dominant CMV-pp65 CTLs were different between cultured and non-cultured cells, especially in Case-3 (Figures 3b and c).

Cytotoxicity

Two clones were established from Case-1: the most dominant clone N-1 with CASSDPGDSLNGYTF of CDR3, and clone N-X with CASSLSGVDYNEQFF, which was not detected by single-cell analysis at any point. Both clone N-1 and clone N-X showed specific cytotoxicity against CMV-pp65 in a dose-dependent manner. The dominant clone N-1 exhibited 53 and 70% of cytotoxicity at the E:T ratio of 1.25 and 5, respectively. On the other hand, the minor clone N-X exhibited 20 and 30% of cytotoxicity at the corresponding E:T ratios (Figures 5a and b).

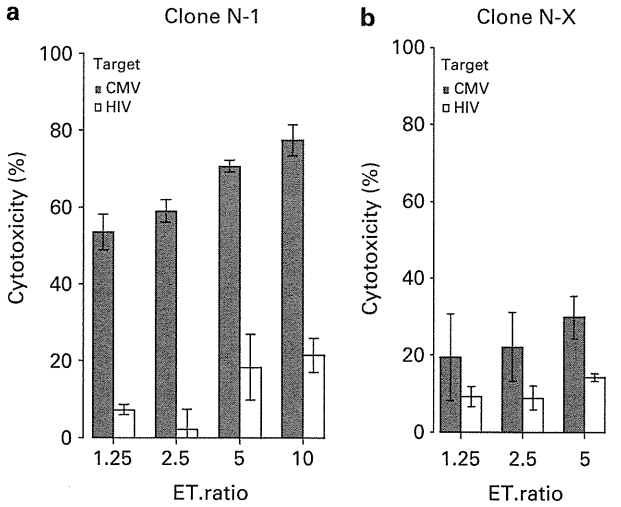

Figure 5. Cytotoxicity assay for clones of HLA-A*2402-restricted CMV-pp65-specific cytotoxic T cells. Two clones were established from Case-1: (**a**) the most dominant clone N-1, with CASSDPGDSLNGYTF of TCR-β CDR3, and (**b**) clone N-X, with CASSLSGVDYNEQFF, which was not found at any point.

DISCUSSION

Using a direct single-cell analysis of the TCR-β repertoire, we conducted clone monitoring of CMV-pp65 CTLs in three donor–recipient pairs of Allo-HCT and analyzed about 1000 cells. The methods enabled the simultaneous identification and quantification of CTL clones and we could visualize the *in vivo* dynamics of CMV-pp65 CTL clones without *in vitro* expansion, which may affect the proportions of CTL clones.

To date, various studies have investigated CMV-specific CTLs[6–10,16,21,22] and reconstitution after Allo-HCT.[5,23–32] However, neither flow cytometry using a panel of mAbs to the TCR-BV family[7,9,27] nor TCR-BV spectratyping[7,10] could reflect the dynamics of CMV-CTLs at a clone level. Furthermore, *in vitro* culture and bacterial transformation are required in a clonotype-specific PCR method, which has been used to identify and quantify CMV-CTL clones.[7,8,30] Therefore, it might result in a biased selection of CTL clones according to their proliferative potential.[33,34] In fact, we observed differences in dominant clones between cultured and non-cultured donor cells in Cases-2 and -3. The current direct single-cell analysis method is important for evaluating the *in vivo* dynamics of CMV-CTL more precisely.

To the best of our knowledge, this study is the first to describe the comprehensive dynamics and monitoring of CMV-pp65 CTL clones after Allo-HCT. We investigated the clone dynamics in three distinct patterns of CMV reactivation: (1) no CMV reactivation, (2) CMV reactivation only within the early phase and (3) repeated CMV reactivation. Like the results in previous studies,[30,35] several CMV-pp65 CTL clones in the donors were actually detected in the recipients after Allo-HCT. Especially in Case-1 without CMV reactivation, dominant clones of Donor-1 persisted and remained dominant even after Allo-HCT, which might reflect a high proportion of CD27-CD57+ mature CMV-pp65 CTLs in Case-1 at 1 year after Allo-HCT. On the other hand, not all of the clones detected in the recipients after Allo-HCT were present in their corresponding donors. Furthermore, we found that dominant clones in a donor were not always dominant in a recipient after Allo-HCT. In Cases-2 and -3 with CMV reactivation, novel clones appeared and became dominant during the follow-up. The emergence of novel clones might be associated with more CD27+ immature CMV-pp65 CTLs at 1 year after Allo-HCT, especially in Case-3.

The cellular response to CMV is known to be frequently impaired and CMV reactivation often occurs in recipients of solid organ transplant or Allo-HCT, even in the presence of CMV-CTL.[21,26] However, in Case-1, CMV reactivation did not occur despite the long-term use of systemic steroid. One possible explanation for this finding may be the protective phenotype of CMV-CTLs in this patient, compared with those in the other two patients. In Case-1, CMV-pp65 CTLs included about 70% of highly differentiated $CD45RA^+CCR7^-$ T_{EF} cells during follow-up, whereas this value was $<30\%$ in the other two patients. Cytokine production and cytotoxicity are known to increase in association with differentiation, and $CD45RA^+CCR7^-$ T_{EF} cells are believed to have high killing potential.[14,15] The clinical course of CMV reactivation might be affected by the phenotype of CMV-CTLs in the recipients after HCT.

It remains a matter of debate which is important for eliminating infectious pathogens, TCR diversity or selected clones with high avidity.[36] In the previous report, TCR diversity was linked to host resistance against viral infection.[37] On the other hand, another report suggested that TCR diversity had no functional advantage on CTL response.[38] In the current study, Case-1 with highly selected clones seemed to have more advantages on CMV reactivation than Case-3 with more diverse repertoire, although this study is too small to draw a conclusion. Further investigation is necessary to address this issue in allo-HCT recipients.

In previous reports on healthy subjects with HLA-A2 or HLA-B7, dominant clones selected specific BV and BJ genes, and had a particular common AA motif of TCR-β CDR3 in unrelated subjects.[9] In the current study, HLA-A*2402-restricted CMV-pp65 CTLs tended to select BV7 and BJ1-1. Although no common AA motif was found among the cases, GGGG was present in dominant clones in two cases. A larger study will be required to show whether a common motif exists in allo-HCT recipients. Effective immune reconstitution is critical to control CMV reactivation before it progresses to CMV diseases such as pneumonia or colitis, which have high mortality rates.[1–4] Gauging the TCR repertoire and identifying most effective clonotypes of CMV-CTLs may lead to a specific immunotherapy against treatment-refractory CMV infection, as in anticancer therapy.[39]

This study had several limitations and challenges regarding the methodology. First, our study included only three donor–patient pairs, although the clonotypes of about 1000 cells were analyzed. Therefore, we could not form any definite conclusions regarding the association between clinical CMV reactivation and the dynamics of CMV-pp65 CTL clones.[26,28–30] Second, it should be noted that the proportion of HLA-A*2402-restricted CMV-CTLs was quite low at later time points, especially in Case-3. Phenotypic analysis might be susceptible to the low number. Third, clones of CMV-pp65 CTLs in Cases-2 and -3 could not be established by the current experimental methods. This may have been due to the exposure to steroid or GVHD. To compare the cytotoxicities and cytokine production of CMV-pp65 CTL clones, a method that enables sufficient cell proliferation from a single cell will need to be established.

In summary, we focused on the reconstitution of HLA-A*2402-restricted CMV-pp65 CTLs following allo-HCT, and performed clone monitoring using a single-cell analysis method. HLA-A*2402-restricted CMV-pp65 CTLs tended to select BV7 and BJ1-1. The phenotypes and *in vivo* dynamics of CMV-pp65 CTLs differed according to three distinct patterns of CMV reactivation. A larger study is warranted to assess the association between CMV reactivation and reconstitution patterns of CMV-CTLs. *In vivo* clone monitoring of CMV-CTLs could provide insight into the mechanism of immunological reconstitution following allo-HCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HN designed the study, performed the experiments, analyzed data and wrote the manuscript. YT and R Yamazaki, designed the study and gave their advice about the experimental procedures. MS, KT, KS, R Yamasaki, HW, YI, KK, TM, MA, Shun-ichi K, MK, AT, JK, Shinichi K and JN collected data. YK designed the study, analyzed data and wrote the manuscript.

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ORIGINAL ARTICLE

Soluble interleukin-2 receptor level on day 7 as a predictor of graft-versus-host disease after HLA-haploidentical stem cell transplantation using reduced-intensity conditioning

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Abstract In the present study, we analyzed the kinetics of serum soluble interleukin-2 receptor (sIL-2R) using data from 77 patients undergoing HLA-haploidentical transplantation using reduced-intensity conditioning (RIC), who were at an advanced stage or at high risk for relapse, to clarify the usefulness of sIL-2R as a biomarker of acute graft-versus-host disease (GVHD). Anti-T-lymphocyte globulin and methylprednisolone were used as GVHD prophylaxis. While the median sIL-2R in 38 patients not developing GVHD was suppressed at levels <740 U/ml, sIL-2R in 25 patients developing severe GVHD peaked on day 11 (1,663 U/ml), and thereafter decreased to <1,000 U/ml after day 30. The occurrence of GVHD was not limited to times of high sIL-2R level, but occurred at any time point on the sIL-2R curve. Most patients developing GVHD, however, experienced a higher sIL-2R level early in their transplant course. The combination of RIC and glucocorticoids sufficiently suppressed sIL-2R levels after HLA-haploidentical transplantation. In a multivariate analysis to identify factors associated with GVHD, day 7 sIL-2R >810 U/ml was the only factor significantly associated with the occurrence of severe GVHD ($p = 0.0101$).

Keywords Allogeneic stem cell transplantation · Graft-versus-host disease · Soluble interleukin-2 receptor · Alloreactive response · HLA-haploidentical transplantation

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Introduction

Bone marrow transplantation (BMT) from siblings genotypically matched for human leukocyte antigen (HLA) improves long-term survival in patients with hematologic malignancies [1]. However, more than 70 % of patients who could benefit from allogeneic BMT do not have a matched sibling donor. On the other hand, there is a >90 % chance of promptly identifying an HLA-haploidentical donor within the family; therefore, the number of patients receiving HLA-haploidentical stem cell transplantation (SCT) is gradually increasing [2–6]. The major drawback of HLA-haploidentical SCT is graft-versus-host disease (GVHD). To overcome GVHD after HLA-haploidentical SCT, several breakthroughs in transplant methodology, including drastic ex vivo T cell purging coupled with the use of megadose of stem cells [2], and in vivo T cell purging through the use of anti-T-lymphocyte globulin (ATG) [4, 5, 7], or the use of cyclophosphamide at post-transplant, have been done [6]. We and others have been studying HLA-haploidentical SCT using in vivo T cell purging method using ATG [4, 5, 7]. In this transplant setting, although the severity of GVHD is within a permissible range, GVHD still continues to be the problem, but an appropriate monitoring method of GVHD has not been established yet.

Basically, GVHD is induced by the immunological response of donor T cells. In general, once activated, T cells express the interleukin-2 receptor (IL-2R), consisting of at least three subunits (α, β and γc) on their membrane

[8, 9]. The soluble form of IL-2R is produced by proteolytic cleavage of IL-2Rα, and the release of soluble interleukin-2 receptor (sIL-2R) into the circulation has been found to be proportional to its membrane bound expression [10, 11]. Thus, serum sIL-2R levels reflect the magnitude of the T cell immunological response and are associated with the incidence and severity of GVHD in allogeneic BMT settings. In fact, sIL-2R is reported to be the most reliable biomarker among several useful biomarkers [12].

The role of sIL-2R as a GVHD biomarker has been studied mainly in the transplant settings of HLA-matched myeloablative SCT for patients mostly in complete remission (CR) [12–17]. Reduced-intensity conditioning (RIC), which has been used also in HLA-haploidentical transplant settings, may contribute to the reduction of the incidence and severity of GVHD [18–20]. We and others reported that HLA-haploidentical reduced-intensity conditioning stem cell transplantation (RIST) was useful for patients who did not have a suitable HLA-matched donor [5, 7]; however, there are no reports analyzing whether sIL-2R is still a useful biomarker of GVHD in this transplant setting.

Despite the usefulness of sIL-2R as a GVHD biomarker, transplant-related complications, including severe infection, graft rejection, and hepatic veno-occlusive disease, are known to increase sIL-2R levels [13, 15, 21]. Furthermore, leukemia- or lymphoma-associated elevation of serum sIL-2R levels has been reported [22–25]. The coexistence of these conditions could reduce the value of sIL-2R as a biomarker of GVHD.

Therefore, in the present study, after excluding data of patients with conditions that increase sIL-2R levels other than GVHD, we retrospectively studied the usefulness of sIL-2R as a GVHD biomarker using data from 77 patients, with poor prognosis or in an advanced stage of disease, who underwent HLA-haploidentical RIST.

Patients and methods

Patients

To retrospectively evaluate the role of the sIL-2R level as a biomarker of acute GVHD, we analyzed data from patients who underwent HLA-haploidentical RIST at the Hospital of Hyogo College of Medicine between January 2009 and June 2012. All patients had hematologic malignancies and were at an advanced stage or had a poor prognosis at the time of transplantation.

The inclusion criteria were as follows: donor-type engraftment, survival for at least 30 days after transplantation, the absence of hepatic veno-occlusive disease, and severe infections (CRP >10), including sepsis [13, 15, 21]. Furthermore, to avoid the effect of tumor-associated sIL-2R [22–25], data from patients who showed a tumor-associated increase in sIL-2R >2,000 U/ml before conditioning, which did not decrease to <1,000 U/ml on day 0, were excluded. Consequently, data from 20 % of the total transplant patients were excluded based on the exclusion criteria described above, and we analyzed data from 77 patients who underwent transplantation using a graft from an HLA-haploidentical donor (2–3 antigen-mismatches in GVH direction). The patients' characteristics are shown in Table 1.

Table 1 Patients' characteristics

	GVHD Grade 0	Grade I	Grade II–III
Number of patients	38	14	25
Sex			
Male/female	24/14	5/9	10/15
Age (years)			
Median (range)	42.5 (17–63)	46.5 (20–61)	55 (14–65)
Disease			
AML/MDS	17	10	12
ALL	11	0	3
Lymphoma	6	2	6
Others	4	2	4
Disease status			
Good (CR/RA/CP)	2	0	2
Intermediate (PR/RAEB/AP)	4	1	1
Poor	32	13	22
HLA disparity in GVH direction			
2 antigen	18	9	13
3 antigen	20	5	12
Number of times of transplant			
First	17	7	20
Second or later	21	7	5
Conditioning combination chemotherapy			
Busulfan-containing	8	3	9
Melphalan-containing	26	8	15
Others	4	3	1
TBI			
Containing	25	8	15
Non-containing	13	6	10

Institutional review board approval was obtained for the treatment protocol, and written informed consent was obtained from the patients and their families.

Preparative regimen for transplantation

Sixty-nine patients received a regimen consisting of fludarabine (30 mg/m^2/day on days −9 to −4), cytarabine (2 g/m^2 on days −9 to −6), ATG (thymoglobulin: total

2.5 mg/kg divided on days -3 to -1), and busulfan (4.0 mg/kg/day on days −6 and −5) or melphalan (70 mg/m^2 on days −3 and −2) with or without TBI 3–4 Gy on day 0. The remaining 8 patients received other agents instead of busulfan or melphalan because of chemoresistance. GVHD prophylaxis consisted of tacrolimus and methylprednisolone (mPSL) 1 mg/kg [5]. All patients except 2 received peripheral blood stem cells. T cell depletion was not performed.

Diagnosis of graft-versus-host disease and supportive care

Acute GVHD was graded according to standard criteria [26]. GVHD was treated as previously described [5]. Each patient was isolated in a laminar air-flow room or protective environment room, and standard decontamination procedures were followed. Oral antibiotics (ciprofloxacin, vancomycin, itraconazole or voriconazole) were administered to sterilize the bowel. Patients who were negative for cytomegalovirus (CMV) IgG received blood products from CMV-seronegative donors. Intravenous immunoglobulin was administered at a minimum dose of 100 mg/kg every 2 weeks until day 100. Cotrimoxazole was given for at least 1 year for prophylaxis of *Pneumocystis carinii* infections. Acyclovir was administered at a dose of 1,000 mg/day for 5 weeks after transplantation to prevent herpes simplex virus or varicella-zoster virus infection, and then the agent was continued for at least 2 years at a dose of 200 mg/day. Ganciclovir 7.5 mg/kg divided into 3 doses per day was administered from day −10 to day −3 as prophylaxis for CMV infection.

Measurement of serum sIL-2R

The serum sIL-2R level was monitored from the start of conditioning three times a week until hospital discharge. The serum sIL-2R concentration was evaluated using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) with two murine anti-human sIL-2R antibodies (CELLFREE Human sIL-2R ELISA Kit; Thermo Fisher Scientific Inc., Rockford, IL, USA). The normal sIL-2R level is <534 U/ml.

Statistics

The background levels of serum sIL-2R were decided using data from 38 patients who did not develop GVHD. The difference in sIL-2R levels on day 7 between patients who developed grade 0–I and grade II–III GVHD was analyzed using the Mann–Whitney *U* test. In addition, we determined the appropriate cutoff value of the sIL-2R level on day 7 to discriminate patients with and without severe GVHD through receiver operating characteristic (ROC) analysis, in which sensitivity and specificity were calculated as a function of the cutoff value, (1-specificity) was plotted against the sensitivity, and the area under the ROC curve (AUC) was calculated. Cumulative incidence of GVHD for patients with sIL-2R on day 7 of >810 or <810 U/ml was estimated using the Kaplan–Meier method, treating death and relapse as competing risks. Gray's test was used for comparison of cumulative incidence in the 2 groups. To identify factors associated with GVHD, using variables including the donor source, age, disease status before transplantation, sex, number of times of transplantation, HLA disparity in GVH direction, disease, and day 7 sIL-2R level, univariate and multivariate analyses were performed using the Cox proportional hazards model. Results were considered significant when $p \leq 0.05$. Statistical analyses were performed with EZR [27, 28].

Results

Background level of sIL-2R based on the data from patients who did not develop GVHD

Serum sIL-2R levels were monitored 3 times a week to analyze the relationship between sIL-2R levels and the development of GVHD in detail. We first identified the serum background level of sIL-2R based on data from 38 patients who did not develop acute GVHD. As shown in Fig. 1, sIL-2R was slightly high, but mostly <1,200 U/ml during 2 weeks after transplantation, and thereafter slightly decreased to <1,000 U/ml. The median value of sIL-2R

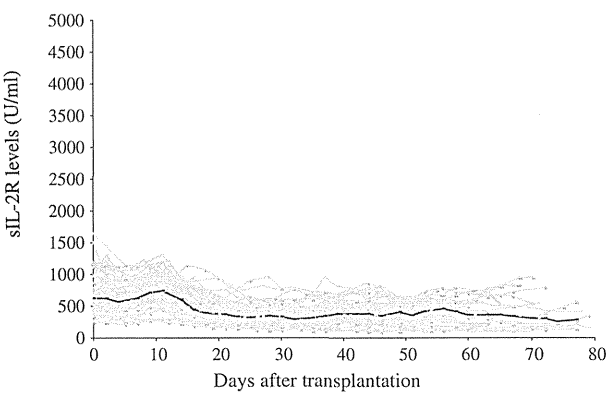

Fig. 1 The kinetics of sIL-2R in patients not developing acute GVHD. To identify the background levels of sIL-2R, changes of serum sIL-2R of 38 patients who did not develop GVHD were plotted. The normal sIL-2R level is <534 U/ml. sIL-2R was slightly high, but mostly <1,200 U/ml during 2 weeks after transplantation, and thereafter slightly decreased to <1,000 U/ml. The *bold line* shows a median sIL-2R level

was slightly increased after transplantation, peaked on day 11 (the peak level was 740 U/ml), and thereafter decreased to levels as low as between 290 and 450 U/ml.

The kinetics of sIL-2R in patients who developed severe GVHD

Next, we analyzed the kinetics of sIL-2R in 25 patients who developed severe (grade II–III) GVHD. Four patients developed skin-only GVHD, 16 gut-only GVHD, and 5 skin/gut GVHD. Those patients developed grade II–III GVHD at a median 28 days (range 3–67 days). The sIL-2R curves were found to vary patient-to-patient in the peak level or in the timing of the peak. The median sIL-2R levels increased after transplantation, reach on day 11 (the peak value of 1,663 U/ml), and thereafter decreased to low levels of <1,000 U/ml after day 30 (Fig. 2).

Regarding the relationship between the kinetics of sIL-2R and the onset of GVHD, 4 patterns were observed. Eight (32 %) patients, in whom sIL-2R increased rapidly after transplantation, developed GVHD at an increasing phase or at the peak level of sIL-2R curve by day 30 (Fig. 3a). These patients developed GVHD at a median of 9 days (range 5–26 days), with the median value of sIL-2R of 1,795 U/ml (range 1,134–4,341 U/ml) at the onset of GVHD. Four (16 %) patients developed GVHD at a decreasing phase of sIL-2R over the peak of sIL-2R (Fig. 3b). These patients developed GVHD at a median of 18.5 days (range 14–21 days), with the median value of sIL-2R of 1,465.5 U/ml (range 618–2,004 U/ml) at the onset of GVHD. Ten (40 %) patients, in whom sIL-2R increased to a high level after transplantation, with the median peak level of 1,711 U/ml (range 1,200–2,977 U/ml) at a median 5.5 days (range 0–16 days), developed GVHD after sIL-2R levels decreased to almost the normal range of sIL-2R (Fig. 3c). These patients developed GVHD at a median of 38 days (range 28–67 days), with the median value of sIL-2R of 642.5 U/ml (range 336–950 U/ml) at the onset of GVHD. Three (12 %) patients, in whom sIL-2R did not increase over the background levels of sIL-2R after transplantation, developed GVHD after day 30, when sIL-2R was slightly increasing over the background level of sIL-2R (Fig. 3d). GVHD in this group of patients occurred latest at a median of 44 days (range 37–59 days), with the median value of sIL-2R of 984 U/ml (range 935–1,054 U/ml) at the onset of GVHD. These results show that GVHD can occur on any point of the sIL-2R curve of patients with GVHD.

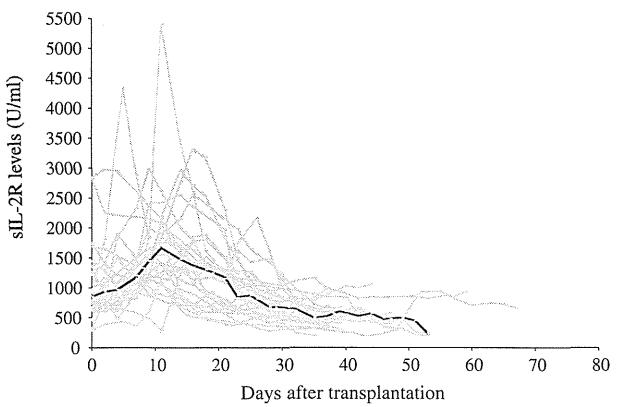

Fig. 2 The kinetics of sIL-2R in 25 patients who developed acute GVHD (grade II–III). Changes of serum sIL-2R in 25 patients who developed severe GVHD were plotted. The sIL-2R curves were found to vary patient-to-patient in the peak level or in the timing of the peak. The *bold line* shows a median sIL-2R level

Prediction of severe acute GVHD by serum sIL-2R levels on day 7

The relationship between sIL-2R change and the onset of GVHD (Fig. 3a–d) shows that the occurrence of GVHD is not limited at the time of high level of sIL-2R or at an increasing phase of sIL-2R; however, the majority of patients who developed severe GVHD showed a high level of sIL-2R early in their transplant course. Therefore, we considered that sIL-2R in the early phase of transplantation may be associated with the development of severe GVHD.

We compared sIL-2R levels on day 7 in patients who developed grade II–III GVHD or grade 0–I GVHD. Consequently, patients with grade II–III GVHD showed significantly higher sIL-2R on day 7 than those with grade 0–I GVHD ($p < 0.0001$). To determine the appropriate cutoff value of sIL-2R on day 7 to discriminate patients with and without severe GVHD, ROC analysis (Fig. 4a) was performed, and the optimal grade II–III GVHD cutoff point was found to be 810 U/ml. The area under the ROC curve (AUC) was 0.790 (CI 0.675–0.904). The relationship between the incidence of severe GVHD and day 7 sIL-2R levels was analyzed using competing risk analysis, treating death and relapse as competing risks. As shown in Fig. 4b, the cumulative incidence of severe GVHD was 43.2 % (CI 28.2–57.3 %) and 6.5 % (CI 1.1–18.9 %) for patients with day 7 sIL-2R >810 U/ml and those with day 7 sIL-2R <810 U/ml, respectively. Patients with day 7 sIL-2R >810 U/ml had a significantly higher risk of GVHD than those with day 7 sIL-2R <810 U/ml ($p = 0.00076$, log-rank test).

Next, using variables including the donor source (offspring vs others), HLA disparity (2 antigen vs 3 antigen) in the GVH direction, older age (>47 years), disease status before transplantation (CR vs non-CR), sex, number of times of transplantation, disease (ALL vs others), and day 7 sIL-2R, factors that were significantly associated with the development of severe GVHD were analyzed using the

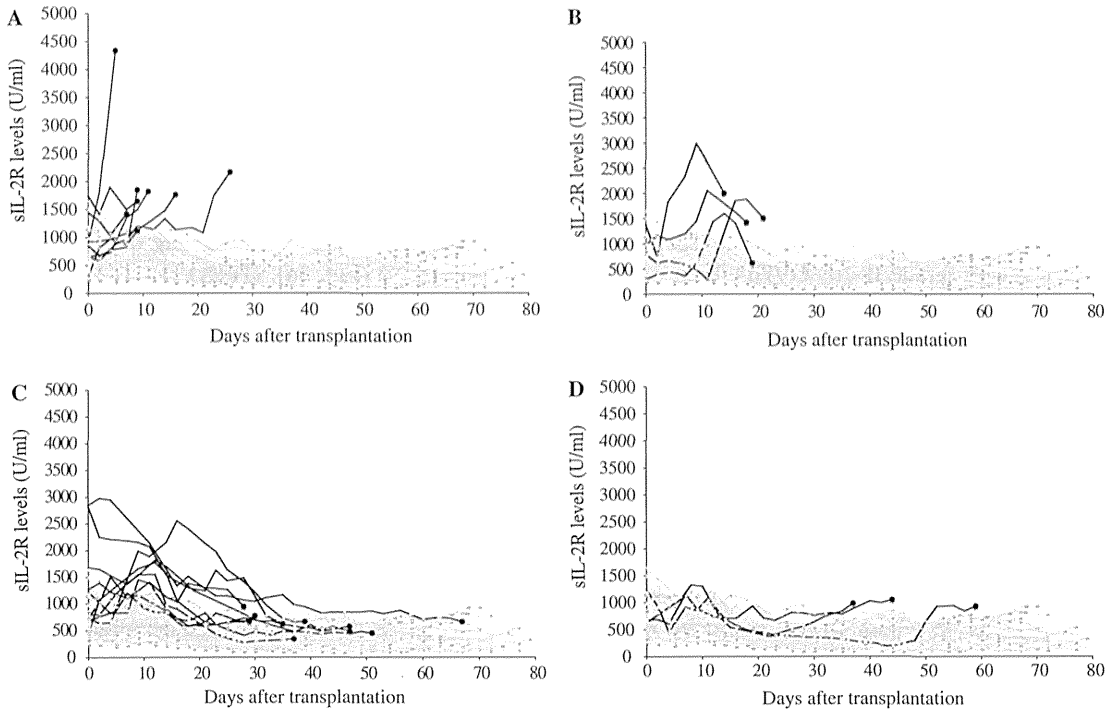

Fig. 3 The relationship between the kinetics of sIL-2R and onset of GVHD in patients who developed severe acute GVHD. *Bold lines* show changes of sIL-2R in patients who developed severe GVHD. *Closed circles*, which are at the end of the *bold lines*, show the onset of GVHD. *Gray lines* show changes of sIL-2R in patients not developing GVHD. **a** GVHD occurred at an increasing phase of sIL-2R or at the peak level by day 30. **b** GVHD occurred at a decreasing phase of sIL-2R (still at a high level) over the peak of sIL-2R. **c** GVHD occurred after returning to the background level of sIL-2R, which passed through the high levels after transplantation. **d** GVHD occurred at a time point slightly increased from the background level of sIL-2R after day 30

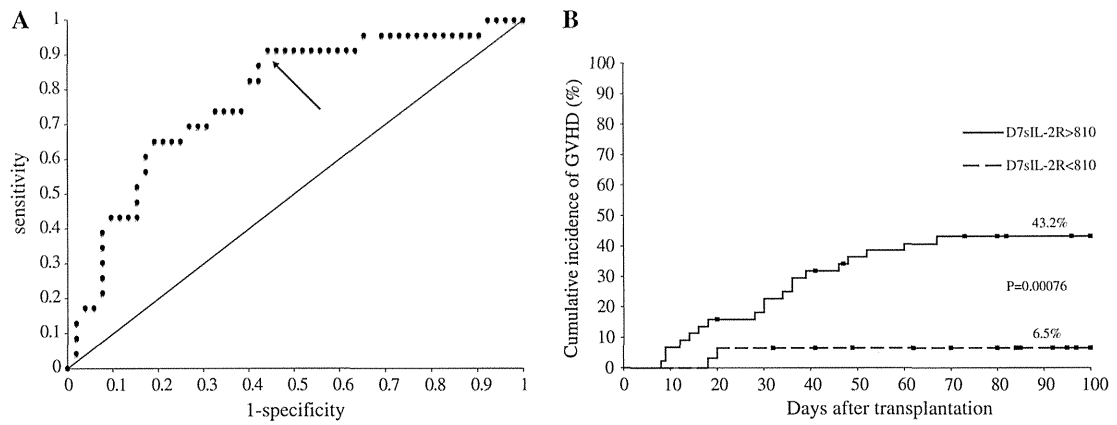

Fig. 4 **a** ROC curve of sIL-2R level on day 7 for the prediction of severe GVHD. To determine the appropriate cutoff value of sIL-2R levels on day 7 to discriminate patients with and without severe GVHD, ROC analysis was performed, and the optimal grade II–III GVHD cutoff point was found to be 810 U/ml, shown by the *arrowhead*. The area under the ROC curve (AUC) was 0.790. **b** Cumulative incidence of severe GVHD for patients with sIL-2R on day 7 of >810 and <810 U/ml. Cumulative incidence of acute GVHD for patients with sIL-2R on day 7 of >810 U/ml and <810 U/ml was estimated using the Kaplan–Meier method, treating death and relapse as competing risks. Gray's test was used for comparison of cumulative incidence in the 2 groups. Patients with sIL-2R on day 7 of >810 U/ml had a significantly higher risk of severe acute GVHD than those with day 7 sIL-2R of <810 U/ml ($p = 0.00076$, log-rank test)

Cox proportional hazards model (Table 2). In a univariate analysis, day 7 sIL-2R >810 U/ml, offspring, age >47 years, and first transplantation were significantly associated with the occurrence of severe GVHD. In a multivariate analysis, day 7 sIL-2R >810 U/ml was only a factor significantly associated with the occurrence of

Table 2 Analysis of factors related to the development of severe GVHD

	Univariate analysis		Multivariate analysis	
	p	CI	*p*	CI
sIL-2R on day 7 (>802 vs <802)	0.0024	2.214–40.431	0.0101	1.597–31.999
Donor source (offspring vs others)	0.0125	1.287–8.102	0.6601	0.238–9.617
Age (>47 vs <47 years)	0.0089	1.389–9.905	0.8337	0.144–11.068
Disease status before transplantation (CR vs non-CR)	0.7203	0.307–5.527	0.1965	0.576–14.591
Sex (female vs male)	0.1877	0.769–3.820	0.526	0.525–3.523
Number of times of transplant (retransplantation vs first transplantation)	0.0155	0.112–0.795	0.2003	0.100–1.621
HLA disparity in GVH direction (2 antigen vs 3 antigen)	0.8831	0.430–2.067	0.5993	0.322–1.922
Disease (ALL vs others)	0.2713	0.152–1.698	0.490	0.181–2.265

CI confidence interval, *CR* complete remission, *non-CR* non-complete remission

severe GVHD ($p = 0.0101$, CI 1.597–31.999). Offspring, age >47 years, and first transplantation had no significant impact on the occurrence of severe GVHD.

Discussion

In the present study, using data from 77 patients who underwent HLA-haploidentical RIST, we investigated the thorough kinetics of serum sIL-2R after transplantation to elucidate the usefulness of sIL-2R as a GVHD biomarker, and found that sIL-2R on day 7 was useful as a predictor of severe GVHD.

In the present study, data from other pathological situations that increase serum sIL-2R were excluded from the analysis. Serum sIL-2R levels reflect the magnitude of the activation and proliferation of T cells, but are not specific to the GVH reaction. This is an inevitable drawback in the diagnosis of GVHD using sIL-2R, as a non-specific T cell reaction of donor or recipient origin produces sIL-2R in some particular transplant complications, such as infection. To avoid the effect of these complications on sIL-2R analysis, other researchers also excluded data from patients with these complications, who represent 15 % of allogeneic recipients [12]. In the present study, a tumor-associated increase in sIL-2R was observed in a slightly high incidence because the majority of patients treated in our institute were in non-CR at the time of transplantation; therefore, data from a slightly higher proportion (20 %) of patients were excluded.

In the absence of GVHD, the median serum sIL-2R was slightly increased after transplantation, peaked on day 11 (the peak level was 740 U/ml), and thereafter decreased to as low as between 290 and 450 U/ml (Fig. 1). On the other hand, in the presence of GVHD, the median serum sIL-2R increased after transplantation, peaked in a median of 11 days (the peak level was 1,663 U/ml), and thereafter decreased to low levels of <600 U/ml (Fig. 2). Compared with the previous studies [14, 16], in which sIL-2R levels peaked 2–3 weeks after transplantation with the peak level of 3,000–5,000 U/ml, sIL-2R in the present study reached the peak level slightly earlier, but the peak levels were lower. The use of ATG-containing RIC regimen and the incorporation of glucocorticoids into the GVHD prophylaxis are considered to contribute to the decrease in the peak level of sIL-2R, which is probably the main reason for a low incidence of severe GVHD observed in our regimen for HLA-haploidentical RIST [5]. Miyamoto et al. [14], in the study of allogeneic SCT using myeloablative conditioning, reported that sIL-2R in patients with GVHD started to increase on day 3, and that the elevation of sIL-2R on day 3 predicted the occurrence of acute GVHD. In the present study, sIL-2R in patients with GVHD started to increase on day 7, as shown in Fig. 2. This discrepancy may be explained by the use of RIC in this study, which induces mixed chimerism status between donor and recipient in the early transplant period, retarding the start of GVH reaction.

The previous studies of sIL-2R only showed that sIL-2R peaked on weeks 2 and 3, or that the peak level of sIL-2R was associated with the severity of GVHD [14, 16]. From the analysis of the onset of GVHD, GVHD was found to occur in 4 different phases of sIL-2R curve: GVHD occurred (1) at an increasing phase or at the peak level of sIL-2R after transplantation (Fig. 3a), (2) at a decreasing phase of sIL-2R (still at a high level) over the peak of sIL-2R (Fig. 3b), (3) after returning to the background level of sIL-2R, which passed through the high levels after transplantation (Fig. 3c), and (4) at a time point slightly increased from the background level of sIL-2R after day 30 (Fig. 3d). Although the prophylactic use of glucocorticoids is considered to contribute to the reduction in sIL-2R levels, as described above, there was no difference among 4 patterns of patients with GVHD in the administration schedule of steroids until the onset of GVHD. The occurrence of GVHD in 4 different phases of sIL-2R curve of

GVHD is not limited to HLA-haploidentical RIST, but observed also in other types of allogeneic SCT, including related HLA- matched, unrelated bone marrow, and umbilical cord blood SCT (data not shown). These results show that GVHD occurs at any time point on the sIL-2R curve, indicating that sIL-2R is not a suitable marker for real-time monitoring of the development of GVHD. Host organ-associated factors [29–31], other than donor T cell activation, must be also associated with the ultimate development of GVHD.

In fact, while sIL-2R peaked at a median of 11 days in patients developing GVHD, GVHD occurred at a median of 28 days. This time lag between the peak level of sIL-2R and the onset of GVHD may be explained as follows. According to the pathophysiology of GVHD that Ferrara et al. [32] proposed, donor T cell activation precedes a series of the subsequent various immunological reactions leading to the development of GVHD. In addition, GVHD may become clinically evident as the dose of immunosuppressive agents is tapered.

On the other hand, the fact that the majority of patients developing GVHD showed a high level of sIL-2R early in their transplant course indicates that sIL-2R levels in the early transplant phase could be a predictor of severe GVHD. In a univariate analysis, day 7 sIL-2R >810 U/ml, offspring, age >47 years, and first transplantation were significantly associated with the occurrence of severe GVHD; however, in a multivariate analysis, day 7 sIL-2R >810 U/ml was only a factor significantly associated with the occurrence of severe GVHD (Table 2). Thus, for the first time, we showed that sIL-2R in the early transplant phase was useful as a GVHD predictor.

The occurrence of events, such as VOD or sepses, until day 7 may result in non-specific increase in sIL-2R on day 7, which make it unable to predict GVHD using day 7 sIL-2R; however, the predictability of GVHD by sIL-2R on day 7 is not basically affected by such pathological events that occur after day 7. Regarding non-specific increase in sIL-2R on day 7, whether we can detect or diagnose such events (inducing increase in sIL-2R) on day 7 is practically important because the GVHD predictor should not be applied if a given patient has such events and shows sIL-2R >810 U/ml. The diagnosis of VOD or severe infections is relatively easy, whereas accurate quantification of residual tumor burden that may lead to tumor-associated increase in sIL-2R may be sometimes difficult. In general, in case of tumor-associated increase, sIL-2R levels are usually high since before transplantation or during conditioning, and tend to gradually or rapidly decrease after transplantation in this early transplant period, whereas, in GVHD-associated increase, sIL-2R levels are increasing at day 7 in most cases, as shown in Fig. 2. Therefore, when applied to patients undergoing allogeneic SCT in CR status, sIL-2R will be more useful as a GVHD predictor. In addition, even if such a non-specific increase blunts the usefulness of sIL-2R as GVHD predictor, when sIL-2R levels are <810 U/ml on day 7, the incidence of GVHD is only 6.5 %, as shown in Fig. 4b, indicating that patients with such low sIL-2R levels have an extremely low risk of developing severe GVHD even in HLA-haploidentical SCT.

In conclusion, in this HLA-haploidentical SCT using the combination of ATG-containing RIC regimen and the incorporation of glucocorticoid into GVHD prophylaxis, sIL-2R levels were mostly suppressed after transplantation compared with other studies on sIL-2R, which possibly lead to a low incidence of severe GVHD. sIL-2R on day 7 was useful as a predictor of GVHD in this transplant setting.

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Conflict of interest The authors declare no conflict of interest.

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Research Article

Recombinant human soluble thrombomodulin attenuates FK506-induced endothelial dysfunction through prevention of Akt inactivation

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ERK1/2

ABSTRACT

Thrombomodulin (TM), a transmembrane glycoprotein on vascular endothelial cells, is a naturally occurring anticoagulant. Recombinant human soluble TM (rTM), composed of the extracellular domain of TM, also shows anti-coagulant and anti-inflammatory activity, but the effects of rTM on microangiopathy remain unclear. We reported that FK506 induced endothelial dysfunction through inactivation of Akt and extracellular-regulated kinase 1/2 using a three-dimensional culture blood vessel model. In the present study, we examined the effects of rTM on FK506-induced endothelial dysfunction. We found that rTM suppressed FK506-induced endothelial cell death, but not the breakdown of capillary-like tube structures. rTM prevented FK506-induced inactivation of Akt, but not of extracellular-regulated kinase 1/2. Akt inhibition by LY294002 abrogated the preventive effect of rTM on FK506-induced Akt inactivation and the suppressive effect of rTM on FK506-induced cell death. These results suggest that rTM attenuates FK506-induced endothelial dysfunction through prevention of Akt inactivation.

Introduction

Thrombotic microangiopathy occasionally develops as life-threatening endothelial dysfunction after solid organ and hematopoietic stem cell transplantations [1]. FK506, a calcineurin inhibitor used for immune suppression, has been shown to be closely associated with post-transplant thrombotic microangiopathy [2]. We have developed a three-dimensional (3D) culture blood vessel model, in which human

Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; DMSO, dimethylsulfoxide; ELISA, Enzyme-linked immunosorbent assay; ERK, extracellular-regulated kinase; HMGB1, high mobility group box1; HUVEC, human umbilical vein endothelial cells; rTM, recombinant human soluble thrombomodulin; SE, standard error; 3D, three-dimensional; VEGF, vascular endothelial growth factor; WST-8, Water tetrazolium salt 8

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umbilical vein endothelial cells (HUVEC) were stimulated with basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) to form and maintain capillary-like tube and lumen structures [3,4]. Angiogenic factors such as bFGF and VEGF are known to activate Akt, which is located downstream of phosphatidylinositol 3-kinase, and extracellular-regulated kinase 1/2 (ERK1/2), a member of mitogen-activatied protein kinase superfamily [5,6]. Using the 3D model, we reported that FK506 induces endothelial dysfunction, which is the breakdown of capillary-like tube structures and endothelial cell death, through inactivation of Akt and ERK1/2 [4].

Thrombomodulin (TM), a transmembrane glycoprotein on vascular endothelial cells, exerts anticoagulant activity through binding of its extracellular domain to thrombin to inhibit fibrin generation. TM also attenuates inflammatory response via several pathways including the degradation of high mobility group box1 (HMGB1), a DNA-binding protein released from dead cells with potent pro-inflammatory function [7,8]. Recombinant human soluble TM (rTM), which is composed of the extracellular domain of TM, shows anti-coagulant and anti-inflammatory activity, and has been approved for the treatment of disseminated intravascular coagulation in Japan [9–11]. In this study, we examined the effects of rTM on FK506-induced endothelial dysfunction.

Materials and methods

Cell culture and reagents

HUVEC isolated from human umbilical cord were purchased from Lonza (Walkersville Inc., MD) and cultured as described previously [4]. Type I collagen solution (Atelocollagen Bovine Dermis, IPC-50), bFGF, and VEGF were obtained from Koken (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), and Humanzyme (Chicago, IL). Other chemicals were purchased from Sigma (St. Louis, MO).

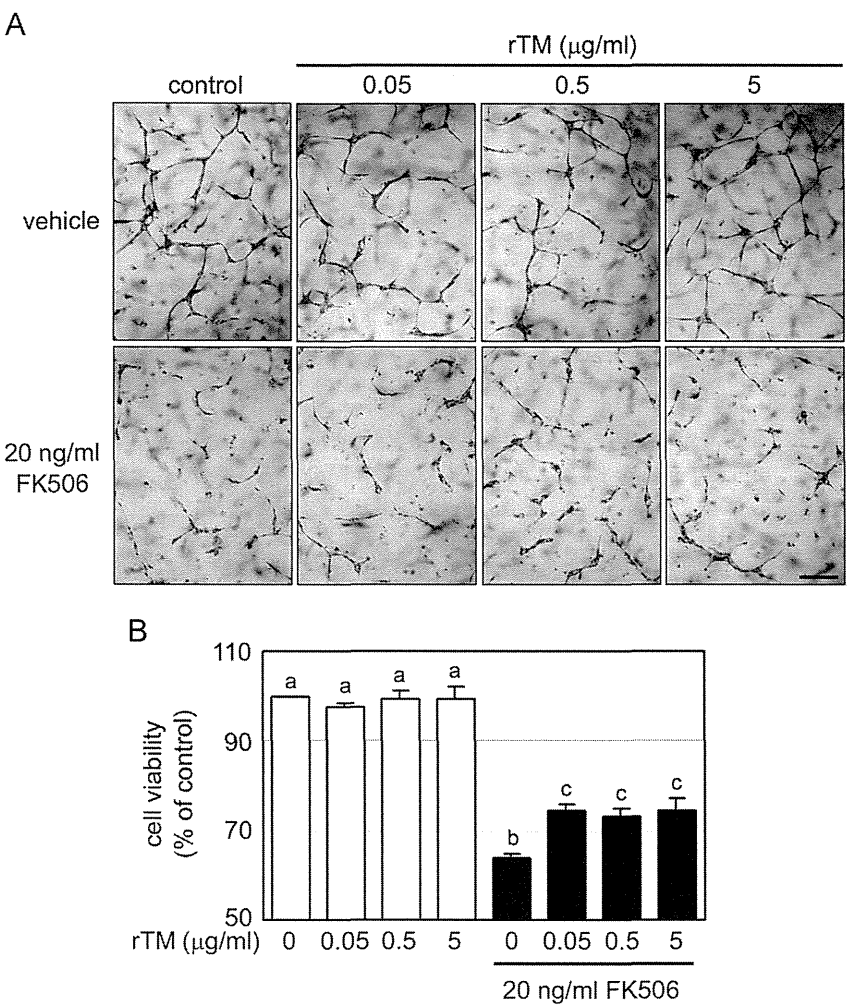

Fig. 1 – Recombinant thrombomodulin (rTM) supresses FK506-induced endothelial cell death in three-dimensional (3D) cultures. (A) rTM fails to suppress FK506-induced breakdown of capillary-like tube structures. Tube-forming HUVEC were incubated with the indicated treatments for 48 h. The bar indicates 100 μm. Representative data of three independent experiments with similar results are shown. (B) rTM suppresses FK506-induced cell death. Tube-forming HUVEC were incubated with the indicated treatments for 48 h, and cell viability was assessed using WST-8 as described in Materials and methods. Values represent means ± SEs of three independent experiments. $^{a,b,c}P<0.05$, values with different letters are significantly different (Bonferroni test).