ORIGINAL ARTICLE

A prospective observational study of immune reconstitution following transplantation with post-transplant reduced-dose cyclophosphamide from HLA-haploidentical donors

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SUMMARY

Allogeneic hematopoietic cell transplantation (HCT) from HLA-haploidentical donors with post-transplantation high-dose cyclophosphamide (PT/Cy-haplo) now predominates worldwide. However, to our knowledge, no prospective study has compared immune reconstitution after PT/Cy-haplo with that after conventional HCT. The mechanism by which chronic graft-versus-host disease (GVHD) is inhibited by PT/Cy-haplo also remains unknown. We prospectively compared immune recovery patterns of lymphocyte subsets among four groups of adult patients with hematological disease who received HCT from either HLA-matched related or HLA-matched unrelated donors, cord blood transplantation, or reduced-dose PT/Cy-haplo. Counts of CD4+ T-cell subsets, CD8+ T-cell subsets, and NK cells on days 30 and 60 were often lower in PT/Cy-haplo than those in HLA-matched related HCT. The immune recovery pace in PT/Cy-haplo subsequently caught up with that of the other grafts. The regulatory T cells (Tregs) to conventional CD4+ T-cell (Tcon) ratio was significantly higher until day 90 in PT/Cy-haplo. In multivariate analysis, a higher Tregs-to-Tcon ratio on day 60 was significantly associated with a lower incidence of chronic GVHD ($P < 0.01$). The preservation of Tregs by PT/Cy in the early phase might have resulted in a lower incidence of chronic GVHD.

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Key words
chronic graft-versus-host disease (GVHD), HLA-haploidentical transplantation, post-transplantation reduced-dose cyclophosphamide (PT/Cy), immune reconstitution, regulatory T cells (Tregs)

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Introduction

Allogeneic hematopoietic cell transplantation (HCT) is still the most effective strategy for cure of refractory hematopoietic malignancies or those with a poor prognosis. In recent years, HLA-haploidentical donors have been increasingly used as an alternative stem cell source in the absence of available HLA-matched donors, especially after the development of HLA-haploidentical hematopoietic cell transplantation using post-transplantation high-dose cyclophosphamide (PT/Cy-haplo) due to its simplicity and favorable regulation of graft-versus-

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host disease (GVHD) [1,2]. PT/Cy administered within a narrow window shortly after transplantation can selectively eliminate host–donor alloreactive T cells, resulting in bi-directional T-cell tolerance and, thus, low incidences of GVHD. In addition, the rate of graft rejection in PT/Cy-haplo is low when compared to that in HLA-haploidentical hematopoietic cell transplantation by stringent *ex vivo* T-cell depletion [3].

There are a few reports on immune reconstitution after PT/Cy-haplo, including those comparing PT/Cy-haplo to HLA-haploidentical HCT by anti-thymocyte globulin (ATG)-containing conditioning regimen [4–7]. However, differences in immune reconstitution patterns between PT/Cy-haplo and after other standard grafts and/or alternative HCTs have not been sufficiently elucidated.

Many prospective studies of PT/Cy-haplo have demonstrated its safety profile and efficacy, irrespective of whether the type of graft used was bone marrow (BM) or peripheral blood (PB) [8–14]. The use of PT/Cy-haplo has become widespread globally, and it is becoming a platform technique for unmanipulated HLA-haploidentical hematopoietic cell transplantation. More recent reports have shown that outcomes in PT/Cy-haplo are comparable to those in HLA-matched related/unrelated transplantation [15]. Moreover, PT/Cy-haplo may have an advantage over HLA-matched unrelated transplantation in terms of the incidence of moderate-to-severe chronic GVHD [15]. However, the underlying mechanism for the low incidence of chronic GVHD with PT/Cy-haplo remains unknown.

To our knowledge, no prospective studies have contemporaneously compared immune reconstitution patterns after HCT among PT/Cy-haplo and other various types of HCTs, including standard HCT. We, therefore, prospectively and comprehensively compared the recovery of lymphocyte subsets among four graft types, including PT/Cy-haplo, and explored the clinical effect of immunological variables on the outcomes.

Methods

The study, performed with the protocol approved by the Ethics Committee of the Graduate School of Medicine, Osaka City University (approval No. 2579), was conducted in accordance with the principles of the Declaration of Helsinki. All patients who were scheduled to receive HCT in the Osaka City University Hospital were eligible to participate in the study. Written informed consent was obtained from each patient.

Patients

Preparative regimen for HCT

The intensity of the conditioning regimens and GVHD prophylaxes are summarized in Table 1. The conditioning regimen for PT/Cy-haplo consisted of fludarabine (150 mg/m^2) and high-dose cytarabine (8 g/m^2)/melphalan (100 mg/m^2). We administered two doses of 25 mg/kg cyclophosphamide on days 3 and 4, after peripheral blood stem cell transplantation (PBSCT). Granulocyte-colony stimulating factor-mobilized PBSCs were used as a stem cell source. With PT/Cy-haplo, the administration of tacrolimus and mycophenolate mofetil (MMF) was initiated following PT/Cy on day 5. If no GVHD occurred until day 40, MMF was discontinued.

As previously described [13], supportive care after HCT was similar for all patients, according to the manual established in the Department of Hematology, Osaka City University Hospital. The detection of viral reactivation/infection and disease and pre-emptive treatments are summarized in the Appendix S1.

Analysis of lymphocyte subsets by multi-color flow cytometry

To define the lymphocyte subsets, fluorochrome-labeled monoclonal antibody cocktails (Table S1) were added to the cell pellet. The following evaluation points included days 30, 60, 90, 180, and 365 after HCT.

The details of the peripheral blood staining procedure, lymphocyte count, and multi-color flow cytometry are described in the Appendix S1, while the gating strategies for the multi-color flow cytometry are included in Figs S1 and S2.

Statistical analysis

Chi–squared tests were used for comparison of categorical values. Kruskal–Wallis tests were used to compare absolute counts and/or ratios, including the percentages of lymphocyte subsets at each evaluation point after HCT. *Post hoc* analysis was conducted using the Steel–Dwass method. Hematopoietic cell transplantation comorbidity index (HCT-CI) and disease risk index (DRI) scores were calculated according to previous reports [16,17]. Acute and chronic GVHD was diagnosed and graded based on clinical manifestations [18–20]. Histological examination of the target site was by biopsy, as possible. The absolute counts and percentages of lymphocyte subsets between patients with and without GVHD in the entire cohort were compared by Mann–Whitney U

Table 1. Patients' characteristics for different donor types: allogeneic hematopoietic cell transplantation (n = 103).

	MRD	MUD	CBT	PT/Cy-haplo	*P*
No. of patients	17	22	25	39	
Median age (range)	45 (24–68)	49 (23–68)	49 (20–68)	48 (21–68)	0.60
Diagnosis (%)					0.19
AML	5 (29)	10 (45)	11 (44)	20 (51)	
ALL	4 (24)	6 (27)	5 (20)	9 (23)	
MDS	2 (12)	3 (14)	0 (0)	1 (3)	
CML	1 (6)	0 (0)	0 (0)	0 (0)	
NHL	3 (18)	3 (14)	7 (28)	9 (23)	
AA	1 (6)	0 (0)	2 (8)	0 (0)	
Others	1 (6)	0 (0)	0 (0)	0 (0)	
DRI (%)					0.06
Low	3 (18)	0 (0)	1 (4)	2 (5)	
Intermediate	6 (35)	13 (59)	9 (36)	10 (26)	
High	5 (29)	4 (18)	9 (36)	19 (49)	
Very high	1 (6)	4 (18)	3 (12)	8 (21)	
NA	2 (12)	1 (5)	3 (12)	0 (0)	
HCT-CI					0.66
0	8 (47)	9 (41)	14 (56)	22 (56)	
<3	4 (24)	8 (36)	8 (32)	8 (21)	
≥3	5 (29)	5 (23)	3 (12)	9 (23)	
Type of source (%)					<0.01*
PB	13 (76)	0 (0)	0 (0)	39 (100)	
BM	4 (24)	22 (100)	0 (0)	0 (0)	
CB	0 (0)	0 (0)	25 (100)	0 (0)	
Donor–patient sex (%)					0.11
F-M	4 (24)	2 (9)	10 (40)	11 (28)	
Others	13 (76)	20 (91)	15 (60)	28 (72)	
Donor/recipient CMV serostatus (%)					<0.01*
D+R+	10 (59)	3 (14)	0 (0)	27 (69)	
D−R+	4 (24)	12 (55)	0 (0)	9 (23)	
D+R−	2 (12)	0 (0)	0 (0)	1 (3)	
D−R−	1 (6)	4 (18)	0 (0)	2 (5)	
NA/R+	0 (0)	3 (14)	21 (84)	0 (0)	
NA/R−	0 (0)	0 (0)	4 (16)	0 (0)	
Conditioning intensity (%)					<0.01*
MAC	10 (59)	16 (73)	14 (56)	1 (3)	
RIC	7 (41)	6 (27)	11 (44)	38 (97)	
GVHD prophylaxis (%)					<0.01*
CSA + sMTX	16 (94)	0 (0)	11 (44)	0 (0)	
CSA + MMF	0 (0)	0 (0)	1 (4)	0 (0)	
TAC + sMTX	0 (0)	22 (100)	0 (0)	0 (0)	
TAC + MMF	0 (0)	0 (0)	13 (52)	39 (100)	
Others	1 (6)	0 (0)	0 (0)	0 (0)	
Times of transplant					0.07
1st transplant	17 (100)	17 (77)	19 (76)	25 (64)	
Beyond 1st transplant	0 (0)	5 (23)	6 (24)	14 (36)	

MRD, allogeneic hematopoietic cell transplantation from HLA-matched related donor; MUD, allogeneic hematopoietic cell transplantation from HLA-matched unrelated donor; CBT, cord blood transplantation; PT/Cy-haplo, HLA-haploidentical hematopoietic cell transplantation with post-transplantation high-dose cyclophosphamide; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; NHL, non-Hodgkin's lymphoma; AA, aplastic anemia; DRI, disease risk index; HCT-CI, hematopoietic cell transplantation comorbidity index; PB, peripheral blood; BM, bone marrow; CSA, cyclosporine A; TAC, tacrolimus; sMTX, short-term methotrexate; MMF, mycophenolate mofetil.

*$P < 0.01$.

tests. Overall survival (OS) and event-free survival (EFS) were estimated using the Kaplan–Meier method and statistically compared by log-rank tests. The incidences of nonrelapse mortality (NRM) or relapse/progression were calculated by Gray's tests. NRM or relapse/progression events were treated as mutually competing events.

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In the analysis of the effect of immunological variables on chronic GVHD for the entire cohort, principal component analysis (PCA) was applied as the first step of the multivariate analysis to compensate for the limit and to address issues related to the large number of variables in the lymphocyte subsets and the low number of patients [21]. The details of PCA are described in the Appendix S1.

A Fine–Gray model was applied to evaluate the effect of clusters of immunological variables (acquired on days 30, 60, and 90) on chronic GVHD. Chronic GVHD was analyzed under the assumption that the risk factors represented competing risks, defining death without chronic GVHD, relapse/progression, and subsequent transplantation as a competing event for chronic GVHD. The confounding factors for chronic GVHD included recipient age, type of graft, donor type, and female donor to male recipient versus others, under consideration of multicollinearity.

All statistical analyses were two-sided, with a *P*-value of 0.05 considered to represent statistical significance. All statistical analyses in the present study were performed using IBM SPSS Statistics for Windows version 20.0 (IBM Corp., New York, NY, USA) and EZR version 1.24 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [22], and a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics

We obtained informed consent from a total of 121 adult patients with hematological disease between October 2013 and November 2016. Eleven of these patients were excluded from evaluation due to early relapse/progression (seven patients), primary graft failure (two patients), graft rejection (one patient), and consent withdrawal (one patient). Finally, 110 patients were included in the analysis of this study. Twenty-one patients received HCT from related donors, 25 received BM transplantation from unrelated donors, 25 received cord blood transplantation (CBT), and 39 received PT/Cy-haplo with PB. The median follow-up duration in survivors in the entire cohort was 678 days (range 133–1405).

Comparisons of the immunity reconstruction patterns of four different grafts

We prospectively compared lymphocyte subset recovery patterns among patients who received HCT from HLA-matched related donors (MRD), HCT from HLA-matched unrelated donors (MUD), cord blood transplantation (CBT), and PT/Cy-haplo (Table 1). Comparison of the PT/Cy-haplo group to the MRD, MUD, and CBT groups revealed higher proportions of patients with high/very high risks of DRI as well as higher proportions of those that underwent reduced-intensity conditioning. The comparison of immune reconstitution recovery patterns excluded patients who received HCT from HLA-mismatched, related, and unrelated donors in order to compare the four different grafts exclusively.

The median follow-up durations in MRD, MUD, CBT, and PT/Cy-haplo survivors were 722 (1273–133), 901 (1405–162), 415 (1902–244), and 657 days (1279–229), respectively. All patients achieved neutrophil engraftment ($\geq 0.5 \times 10^9$/l). The incidences of platelet engraftment ($\geq 20 \times 10^9$/l) were 93% for MRD, 86% for MUD, 90% for CBT, and 90% for PT/Cy-haplo. The median times for neutrophil and platelet engraftment were 15 and 32 days for MRD, 17 and 35 days for MUD, 22 and 58 days for CBT, and 15 and 36 days for PT/Cy-haplo, respectively. Both neutrophil and platelet engraftment were significantly slower for CBT ($P < 0.01$ and $P = 0.02$).

Among patients who received PT/Cy-haplo, naïve, memory T-cell subsets, and NK-cell counts decreased universally in the early phase, which was within 90 days of HCT (Fig. 1). The total CD4+ T-, total CD8+ T-, CD4+ naïve, central memory, and effector memory T-cell counts on days 30 and 60 were significantly lower in the PT/Cy-haplo group than those in the HCT from MRD group.

Day 30 NK-cell and CD8+ naïve T-cell counts and day 60 CD8+ central and effector memory T-cell counts were significantly lower in the PT/Cy-haplo group than those in the MRD group.

In addition, NK- and CD4+ effector memory T-cell counts on day 30 were significantly lower in the PT/Cy-haplo group than those in the MUD group. Days 30 and 60 NK- and CD4+ naïve T-cell counts on day 30 were significantly lower in the PT/Cy-haplo group than those in the CBT group.

In contrast, NK-cell counts on day 90 and day 180 and total B-cell and naïve B-cell counts on day 90 were significantly higher in the CBT group than those in the MRD group (Figs 1 and 2). NK-cell counts on days 30 and 60; total B-cell counts on days 30, 60, and 90; naïve B-cell counts on days 60 and 90; and memory B-cell counts on day 30 were also significantly higher in the CBT group than those in the PT/Cy-haplo group. Moreover, the percentage of recent thymic emigrant (RTE) Treg on day 30

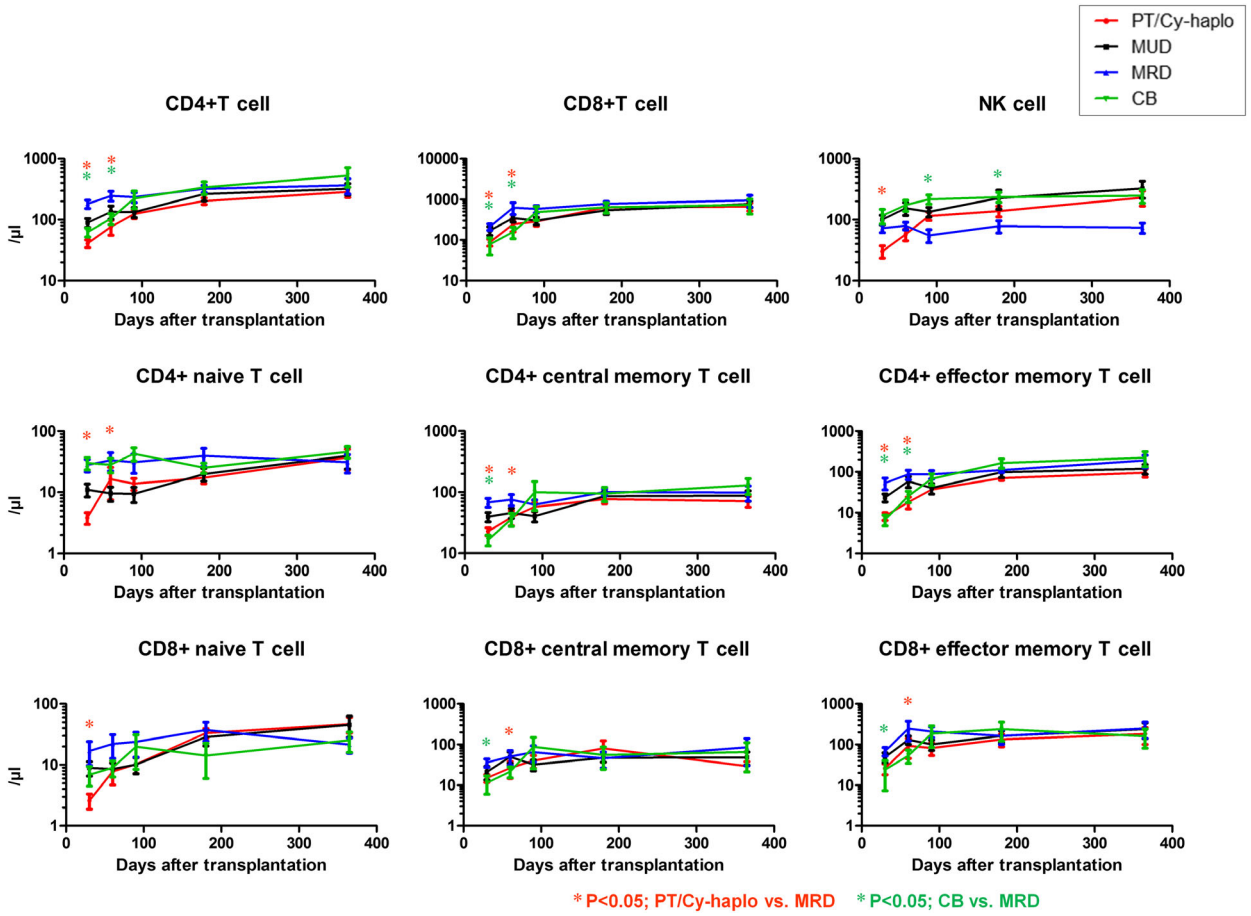

Figure 1 Comparisons of T-cell subsets and NK-cell reconstitution in hematopoietic cell transplantation from MRD, MUD, CBT, and PT/Cy-haplo. Longitudinal analysis of the absolute numbers of T-cell subsets and NK cells following allogeneic hematopoietic cell transplantation. Data are means ± standard error of the mean. Kruskal–Wallis tests were conducted, and *post hoc* analysis was performed using the Steel–Dwass method. MRD, HLA-matched related donor; MUD, HLA-matched unrelated donor; CBT, cord blood transplantation; PT/Cy-haplo, HLA-haploidentical allogeneic hematopoietic cell transplantation with post-transplantation high-dose cyclophosphamide.

was significantly higher in the CBT group than in the MRD and PT/Cy-haplo groups (Fig. 2).

Conversely, no effects were seen on B-cell subset and regulatory T cell (Tregs) counts in the PT/Cy-haplo group, resulting in a significantly higher ratio of Tregs to conventional CD4+ T cells (Tcon) in the PT/Cy-haplo group from days 30–90 than those in the other groups (Fig. 2).

In the PT/Cy-haplo group, the Vδ2+ T cell counts from days 30–90 were significantly lower than those in the MRD and the MUD groups. Days 30 and 90 invariant NKT-cell counts were significantly lower than those in the MRD and CBT groups (Fig. 2).

Comparisons of outcomes among four different grafts

The 2-year probabilities of OS in the MRD, MUD, CBT and PT/Cy-haplo groups were 74%, 53%, 54%, and 43%, respectively. The 2-year probabilities of OS in the PT/Cy-haplo group tended to be lower compared to those in the MRD group ($P = 0.09$).

The 2-year probabilities of EFS in the MRD, MUD, CBT, and PT/Cy-haplo groups were 47%, 50%, 35%, and 32%, respectively.

The 2-year incidences of NRM and relapse/progression in the MRD, MUD, CBT, and PT/Cy-haplo groups were 12%, 22%, 24%, and 14% and 31%, 27%, 40%, and 53%, respectively. There was no statistical difference between the four groups.

Incidences of viral reactivation/infection and disease

CMV high-risk patients, defined as positive recipient serostatus, comprised 82% of patients in the MRD, 82% of MUD, 84% of CBT, and 92% of PT/Cy-haplo groups, respectively ($P = 0.15$). CMV antigenemia was observed in 59% of patients in the MRD, 68% in the

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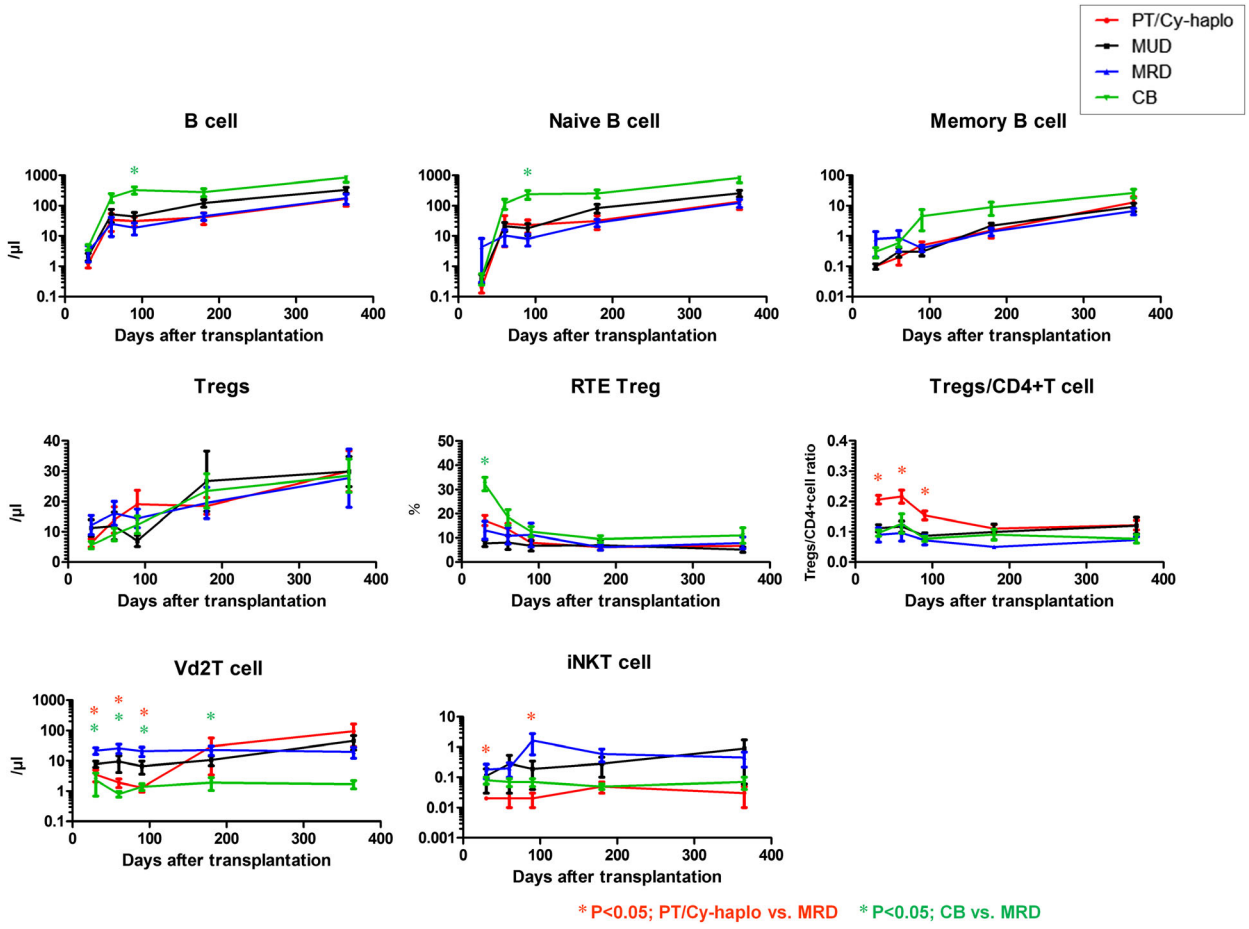

Figure 2 Comparisons of counts of total B cells, B-cell subsets, and Tregs; RTE Treg reconstitution; and changes in the Tregs/CD4+ T-cell ratio in hematopoietic cell transplantation from MRD, MUD, CBT, and PT/Cy-haplo. Longitudinal analysis of the absolute numbers of total B cells, B-cell subsets, Tregs RTE Treg reconstitution, and changes in the Tregs/CD4+ T-cell ratio following allogeneic hematopoietic cell transplantation. Data are means ± standard error of the mean. Kruskal–Wallis tests were conducted, and *post hoc* analysis was performed using the Steel–Dwass method. MRD, HLA-matched related donor; MUD, HLA-matched unrelated donor; CBT, cord blood transplantation; RTE Treg, recent thymic emigrant-regulatory T cell; iNKT, invariant NKT cell; PT/Cy-haplo, HLA-haploidentical allogeneic hematopoietic cell transplantation with post-transplantation high-dose cyclophosphamide.

MUD, 60% in the CBT, and 72% in the PT/Cy-haplo groups ($P = 0.70$).

The occurrences of symptomatic CMV reactivation/infection and disease are shown in Table 2. There were no significant differences in the incidence of symptomatic CMV reactivation/infection and disease between the four groups (Table 2). Treatment with ganciclovir and valganciclovir including pre-emptive therapy or foscarnet was administered in 53%, 64%, 76%, and 79% of patients in the MRD, MUD, CBT, and PT/Cy-haplo groups, respectively ($P = 0.18$). There was also no significant difference in the incidence of symptomatic Epstein–Barr virus (EBV) reactivation/infection and disease ($P = 0.21$). Treatment with rituximab for EBV infection and disease was 0% in both MRD and MUD groups, 12% in the CBT group, and 5% in the PT/Cy-haplo group ($P = 0.19$).

In terms of other viruses, the incidences of symptomatic reactivation/infection and disease are also summarized in Table 2. The MRD group showed a trend of a lower frequency of symptomatic reactivation/infection and disease overall, despite the lack of statistically significant differences, except for BK polyomavirus (BKV, Table 2). The incidences of symptomatic BKV reactivation/infection and disease were higher in the CBT and PT/Cy-haplo groups (Table 2).

Comparisons of the incidences of GVHD among different grafts

The day 100 cumulative incidences of grades II-IV acute GVHD in the MRD, MUD, CBT, and PT/Cy-haplo groups were 47%, 46%, 72%, and 62%, respectively

($P = 0.28$). The 2-year cumulative incidences of chronic GVHD in these groups were 35%, 16%, 18%, and 8.5%, respectively ($P = 0.09$). The 2-year incidences of chronic GVHD tended to be lower in the PT/Cy-haplo group than with other grafts. Mild, moderate, and severe chronic GVHD occurred in 13%, 53%, and 33%, respectively, among patients who met the criteria for chronic GVHD from the National Institutes of Health.

Comparisons of immunological parameters between patients with and without GVHD in the entire cohort

In the analysis including the entire cohort, only memory B-cell counts on day 30 was significantly lower in patients with acute GVHD, than that in those without (Table S2). The lower percentages of CD4+ and CD8+ naïve T cells on days 60 and 90; lower Tregs-to-Tcon ratio on days 30 and 60; and lower percentage of RTE Treg on day 60 were significantly correlated with the occurrence of chronic GVHD (Table 3 and Table S3).

Moreover, higher total CD8+ T-cell and CD8+ effector memory cell counts on day 30 were significantly related to the occurrence of chronic GVHD. Higher CD8+ effector memory T-cell counts on day 90 were significantly associated with the occurrence of chronic GVHD (Table 3 and Table S4).

Clusters of immunological parameters associated with the risk of chronic GVHD in the entire cohort

In this analysis, PCA identified two clusters of variables that correlated with chronic GVHD. In the multivariate analysis, lower ratio of Tregs-to-Tcon (<0.12) and higher CD8+ T-cell counts (>51/μl) on day 30, a lower ratio of Tregs-to-Tcon (<0.13) on day 60, higher CD8+ effector memory T-cell counts (>30/μl), and lower percentage of CD8+ naïve T cell (<4.0%) on day 90 were all significant risk factors of chronic GVHD (multivariate model 1 in the Table 4). Conversely, a higher ratio of Tregs-to-Tcon on day 60 significantly reduced the risk of chronic GVHD (HR per % 0.86, 95% confidential interval 0.79–0.95; $P < 0.01$), irrespective of the donor type (multivariate Model 2 on Table 4).

Discussion

Our prospective observational study showed that the recovery patterns of T-cell subsets and NK cells following PT/Cy-haplo and CBT were quite distinct when compared to those of HCT with a standard graft. Our results also showed that the retentive effect of PT/Cy-haplo on Tregs resulted in a high Tregs-to-Tcon ratio in the early phase. Although many studies have demonstrated the relatively low incidence of chronic GVHD in PT/Cy-haplo, the mechanism by which alloreactive T-cell eradication immediately after HCT with PT/Cy-haplo leads to the suppression of chronic GVHD has not been elucidated. In our results, a high ratio of Tregs to Tcon in the early phase (median ratios: 0.21 and 0.22 on days 30 and 60, respectively, in the PT/Cy-haplo group) was significantly related to a low incidence of chronic GVHD irrespective of PT/Cy-haplo use. We, therefore, speculated that the preservation of Tregs by PT/Cy-haplo might have the potential to suppress the activation of T cells including alloreactive T cells. Thus,

Table 2. Symptomatic reactivation/infection and disease *n* (%).

Type of virus	MRD $N = 17$	MUD $N = 22$	CBT $N = 25$	PT/Cy-haplo $N = 39$	P
HSV	0 (0)	3 (14)	1 (4)	1 (3)	0.17
HHV6	0 (0)	5 (23)	4 (16)	3 (8)	0.12
CMV	1 (6)	5 (23)	9 (36)	8 (21)	0.14
EBV	0 (0)	1 (5)	4 (16)	6 (15)	0.21
VZV	0 (0)	0 (0)	2 (8)	3 (8)	0.36
ADV	0 (0)	1 (5)	1 (4)	0 (0)	0.48
Influenza A	0 (0)	0 (0)	0 (0)	2 (5)	0.34
BKV	1 (6)	3 (14)	10 (40)	12 (31)	0.037*
JCV	0 (0)	0 (0)	0 (0)	1 (3)	0.65

MRD, HLA-matched related allogeneic hematopoietic cell transplantation; MUD, HLA-matched unrelated allogeneic hematopoietic cell transplantation; CBT, cord blood transplantation; PT/Cy-haplo, HLA-haploidentical hematopoietic cell transplantation with post-transplantation high-dose cyclophosphamide; HSV, herpes simplex virus; HHV6, human herpesvirus 6; CMV, cytomegalovirus; EBV, Epstein–Barr virus; VZV, varicella zoster virus; ADV, adenovirus; BKV, BK polyomavirus; JCV, JC polyomavirus.

*$P < 0.05$.

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Table 3. Summary of association between proportion and counts of lymphocyte subsets, and occurrence of chronic GVHD in the entire cohort.

(%)	Day 30	*P*	Day 60	*P*	Day 90	*P*	(cells/μl)	Day 30	*P*	Day 60	*P*	Day 90	*P*
CD4+ T		0.41		0.39		0.30	CD4+ T		0.50		0.69		0.54
CD8+ T		0.14		0.06		0.10	CD8+ T	↑	0.046*		0.07		0.06
NK		0.20		0.29		0.63	NK		0.07		0.93		0.77
CD4+ naïve T		0.40	↓	0.03*	↓	0.04*	CD4+ naïve T		0.16		0.85		0.44
CD4+ CM T		0.62		0.60		0.62	CD4+ CM T		0.47		0.62		0.59
CD4+ EM T		0.50		0.09		0.12	CD4+ EM T		0.61		0.28		0.31
CD8+ naïve T		0.08	↓	0.02*	↓	0.04*	CD8+ naïve T		0.32		0.59		0.68
CD8+ CM T		0.84		0.90		0.80	CD8+ CM T		0.06		0.16		0.18
CD8+ EM T		0.06		0.31		0.33	CD8+ EM T	↑	0.03*		0.10	↑	0.04*
iNKT		0.18		0.28		0.83	iNKT		0.97		0.73		0.93
Vδ2+ T		0.30		0.65		0.56	Vδ2+ T		0.06		0.20		0.97
Tregs/Tcon (ratio)	↓	0.02*	↓	0.007*		0.30	Tregs		0.43		0.20		0.62
RTE Treg		0.54	↓	0.04*		0.34	RTE Treg		0.24		0.98		0.96
B		0.36		0.39		0.13	B		0.74		0.95		0.68
Naïve B		0.39		0.24		0.61	Naïve B		0.77		0.94		0.85
Memory B		0.70		0.30		0.56	Memory B		0.34		0.76		0.81

↑ indicates significant increased value ($P < 0.05$) and ↓ significant decreased value ($P < 0.05$) in patients with chronic GVHD.

*$P < 0.05$.

GVHD, graft-versus-host disease; NK, natural killer; CM T, central memory T cells; EM T, effector memory T cells; Vδ2 + T, Vδ2 + T cells**;** iNKT, invariant NKT cells; Tregs, regulatory T cells; Tcon, CD4+ conventional T. CD4+ T cells; RTE Tres, recent thymic emigrant-regulatory T cell. CD4+ T cells, CD8+ T cells, NK cells, and B cells are represented as the proportion (%) of total lymphocytes. CD4+ naïve, central, and effector memory T cells were calculated as proportion (%) of CD4+ T cells. CD8+ naïve, central, and effector memory T cells were calculated as proportion (%) of CD8+ T cells. Tregs/Tcon and RTE Treg are represented as ratio of CD4+ T cells and Tregs, respectively. Naïve and memory B cells were calculated as proportion (%) of CD19+ CD20+ B cells.

Table 4. The clusters of immunologic parameters related to the incidence of chronic GVHD.

	Day 30	*P*	Day 60	*P*	Day 90	*P*
Clusters	Tregs/Tcon < 0.12 and CD8+ T > 51/μl		Tregs/Tcon < 0.13		CD8+ EM > 30/μl and CD8+ naïve T < 4.0%	
Univariate model	HR 9.4 (95% CI: 1.8–47)	$P < 0.01$	HR 9.6 (95% CI: 1.3–70)	$P = 0.03$	HR 6.0 (95% CI :1.6–23)	$P < 0.01$
Multivariate model 1	HR 12 (95% CI: 2.4–64)	$P < 0.01$	HR 8.5 (95% CI: 1.1–65)	$P = 0.04$	HR 7.4 (95% CI :1.8–30)	$P < 0.01$
Multivariate model 2			HR per % 0.86 (95% CI: 0.79–0.95)	$P < 0.01$		

In the multivariate model 2, Tregs/Tcon (%) was treated as a continuous variable.

HR, hazard ratio, CI, confidential interval.

Confounding factors: Age, type of source, donor type, and female to male transplant.

our results indicate the possible durable suppressive effects of PT/Cy-haplo on the production of alloreactive T cells due to the preservation of Tregs by PT/Cy-haplo in the early phase, which might be involved in the suppression of the chronic alloreactive response in addition to alloreactive T-cell eradication. Indeed, a recent experimental study in an NOD/Lt-scid/IL-2rγ^{null} murine xenogeneic model using Treg-depleted human PB

mononuclear cells demonstrated that PT/Cy-haplo regulated GVHD depending on Tregs [23]. This report also demonstrated that aldehyde dehydrogenase plays a crucial role due to the effects of PT/Cy-haplo to preserve Tregs.

In contrast, at the late evaluation point (day 90), close to the onset of chronic GVHD, increased CD8+ effector memory T-cell counts were associated with the risk of chronic GVHD. This result is concordant with previous reports showing that memory T cells principally contribute to the dysregulated and skewed immune system in chronic GVHD [21,24,25]. There is a report that the naïve T-cell population increased at 3 months after HCT in patients with chronic GVHD [26]. However, an early increase in naïve T-cell proportions was not observed in the chronic GVHD patients in our study. The reason for this difference is unknown but might have been due to differences in the transplantation process, including PT/Cy-haplo and GVHD prophylaxis and/or the use of steroids. By reacting to allogeneic antigens, activated naïve T cells might switch to effector memory T cells, leading to chronic GVHD. As a compensatory mechanism, the number of CD8+ naïve T cells might be decreased in patients with chronic GVHD [25].

Following HCT, with the exception of PT/Cy-haplo, many investigators have evaluated the effect of immune reconstitution on the risk of chronic GVHD in prospective and serial measurements of immunological cell subsets, [27–36]. However, these results were relatively controversial. One reason may be that many investigators focus on limited immunological subsets such as Tregs, dendritic cells, or invariant NK cells, without evaluating immunological parameters collectively. Another possible reason may be the inconsistent and complicated interaction among many immunological indicators during reconstitution. We, therefore, collectively evaluated lymphocyte subsets and applied PCA to reduce the noise due to interaction among variables [21].

In the present study, T-cell subset and NK-cell counts were universally low on day 30 and/or day 60 following PT/Cy-haplo. The delayed recovery of T-cell subsets and NK cells in the PT/Cy-haplo group likely reflects the selective depletion of proliferating alloreactive T and NK cells by PT/Cy-haplo [37]. The elaborate clinical analysis by the investigators of the Italian group demonstrated that naive-derived T memory stem cells that survived PT/Cy administration played a crucial role in T-cell reconstitution after PT/Cy-haplo by generating memory T cells in response to exogenous antigens [38]. The reports indicated that low counts in lymphocyte subsets did not reflect poor immune reconstitution in the PT-Cy-haplo group, in contrast to other HCT settings. Comparison of the pace of immune recovery between MUD and PT/Cy-haplo groups revealed that some T-cell subsets and NK cells in the early phase were significantly slower in the PT/Cy-haplo group than those in the MUD group. However, the pace of immune recovery in T-cell subsets and NK cells in the PT/Cy-haplo group caught up to those in the other groups and were comparable after 3 months.

Furthermore, the recovery pattern of immune cells in CBT was also unique. In the early phase after HCT, the counts of memory T cells but not naïve T cells were similarly low in the PT/Cy-haplo group. In CBT, the pace of recovery of NK and B cells was rapid and the proportion of RTE Treg became high soon after HCT compared to those in PT/Cy-haplo. However, these findings were not associated with GVHD. The rapid recovery of RTE Treg and B cells, especially naive B cells, in the CBT group likely indicated that immature cells contained in CB grafts are more abundant than in PB and/or BM.

Our study had several limitations. We previously performed a clinical trial with a reduced dose of post-transplantation cyclophosphamide to induce graft-versus-leukemia effects, and we found in the prospective study that HLA-haploidentical T-cell replete PBSCT with 25 mg/kg × 2 doses of PT/Cy might be a feasible option [13]. Therefore, the current prospective study also administered this reduced dosage and double doses of 25 mg/kg PT/Cy. The immune recovery patterns might differ among patients in the PT/Cy-haplo group depending on the dosage of PT/Cy. We speculated that the double doses of 50 mg/kg of PT/Cy could increase the Tregs-to-Tcon ratio by more effectively eradicating alloreactive T cells. Indeed, the incidence of acute GVHD was high compared to that in patients receiving the original dose of PT/Cy [4,8–12]. Nevertheless, the incidence of chronic GVHD was very low, suggesting that the two doses of 25 mg/kg PT/Cy were sufficient to regulate chronic GVHD.

In addition, only four of 110 patients (MRD, 1; MUD, 1; CBT, 2) who received ATG-containing conditioning were included in the comparison analysis of immune reconstitution. Thus, the use of ATG was not likely to have significantly affected the result because of the small population.

Furthermore, for a long time, only BM was available for unrelated HCT in Japan. For HCT from the Japan Marrow Donor Program, BM alone was used in all patients in our cohort. Moreover, as our study was merely observational and not an interventional clinical

trial, we did not completely exclude other confounding factors that might have influenced the incidence of chronic GVHD.

In conclusion, the results of this prospective study highlighted the possibility that PT/Cy administered shortly after HCT generates a high Tregs-to-Tcon ratio in the early phase after HCT, which might have contributed to the induction of immune tolerance.

Authorship

HN, SN, HO, TN, HK, YN, AH, MN, TT, and MH: developed the protocol. HN, SN, HO, TN, HK, YN, and MH: collected samples. HN and KF: analyzed flow cytometry samples. HN: wrote the first version of the manuscript. All authors interpreted data and revised the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest

HN received research funding from Astellas Pharma and honoraria from Astellas Pharma and Chugai Pharmaceutical Co. TN and SN received honoraria from Chugai Pharmaceutical Co. HK received research funding from Chugai Pharmaceutical Co. and honoraria from Astellas Pharma. YN received research funding and honoraria from Astellas Pharma. MH received research funding from Astellas Pharma, honoraria from Chugai Pharmaceutical Co., and unrestricted donations to assist in university studies from Chugai Pharmaceutical Co. and Astellas Pharma. This study was not supported by pharmaceutical companies and no patents or copyright exist for this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. The methods of detection of viral reactivation/infection and disease, multi-color flow cytometry analysis and principal component analysis.

Table S1. The fluorochrome-labeled monoclonal antibody cocktails.

Table S2. Association between proportion and counts of lymphocyte subsets, and occurrences of acute GVHD in the entire cohort.

Table S3. Association between proportion of lymphocyte subsets, and occurrences of chronic GVHD in the entire cohort.

Table S4. Association between counts of lymphocyte subsets, and occurrence of chronic GVHD in the entire cohort.

Figure S1. The gating strategies for the multi-color flow cytometry 1.

Figure S2. The gating strategies for the multi-color flow cytometry 2.

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