Optimized Timing of Post-Transplantation Cyclophosphamide in MHC-Haploidentical Murine Hematopoietic Cell Transplantation

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Abstract
Post-transplantation cyclophosphamide (PTCy) reduces the risks of severe acute and chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic cell transplantation (HCT). Yet, the standard clinical dose and timing of PTCy were partly extrapolated from MHC-matched skin allografting models and were partly empirical. Here we investigated the impact of differential dosing and timing of PTCy on its efficacy in preventing GVHD in a murine MHC-haploidentical HCT model. Administration of PTCy on days +3/+4 was superior to administration on days +1/+2, +5/+6, or +7/+8, whereas low-dose (10 mg/kg/day) PTCy on days +1/+2 actually led to accelerated death. Although the optimal timing of PTCy dosing was day +2 or +3 in the skin allografting models, in our MHC-haploidentical HCT model, PTCy on days +2/+3 was inferior to PTCy on days +3/+4 at lower doses. PTCy administered on days +3/+4, +4/+5, or +3/+5 were similarly efficacious. Single-day versus 2-day dosing schedules demonstrated that PTCy is maximally effective when given on day +4. Flow cytometric analysis showed that optimal PTCy dosing schedules both decreased alloreactive CD4⁺CD25⁺ Foxp3⁻ T cell proliferation at day +7 and allowed preferential CD4⁺CD25⁺Foxp3⁺ T cell reconstitution at day +21, suggesting that this combination may be a potential predictive biomarker of successful GVHD prevention by PTCy. These results show that the dose, timing, and cumulative exposure of PTCy all are critical for its efficacy in preventing GVHD. We are currently investigating the clinical relevance of these findings in a protocol seeking to optimize PTCy dose and timing and test these T cell endpoints as candidate biomarkers of successful GVHD prevention by PTCy. Published by Elsevier Inc. on behalf of the American Society for Transplantation and Cellular Therapy.

INTRODUCTION
The use of post-transplantation cyclophosphamide (PTCy) has overcome historical barriers to hematopoietic cell transplantation (HCT) by safely facilitating HLA-haploidentical HCT and by reducing the rates of severe acute and particularly chronic graft-versus-host disease (GVHD) [1]. The clinical implementation of PTCy was based on studies in MHC-matched skin allografting models, which suggested that a high 200 mg/kg dose of PTCy on day +2 or +3 after donor splenocyte infusion was effective in prolonging survival of a subsequently placed skin allograft; lower doses or administration at earlier or later times were less effective or ineffective [2]. When PTCy was adapted to HCT preclinically, 200 mg/kg PTCy on day +2 or +3 promoted engraftment of MHC-mismatched murine allografts and could decrease GVHD [3,4].

PTCy then was translated to the clinic to try to overcome the historical barriers to HLA-haploidentical HCT, namely the high rates of graft rejection and severe GVHD associated with HLA-mismatched allografting [5,6]. For the first clinical study, PTCy was given at 50 mg/kg on day +3 [7]. This dosage was chosen because 1) a “high dose” was required in the MHC-matched skin allografting models, and 50 mg/kg is nearly the maximal tolerable dose in humans and had proven effective as high-dose therapy for aplastic anemia [8], and 2) since days +2 and +3 were equivalent in the skin allografting models [2], this timing would maximize the delay between conditioning and PTCy to minimize toxicity.

Based on promising results from the phase I study [7], a phase II study was initiated at 2 institutions. One institution administered the same PTCy dosage (50 mg/kg on day +3), whereas the second institution also administered a second PTCy dose of 50 mg/kg on day +4 [9]. This empiric change was made in an attempt to further improve GVHD and engraftment outcomes. A cross-cohort, cross-institutional comparison suggested that the days +3/+4 dosing schema may be associated with better outcomes. An understanding of the optimal dosing and timing of PTCy is critical for further improving GVHD prevention in the MHC-haploidentical HCT setting.
with a lower risk of extensive chronic GVHD [9]. Based on these limited data, nearly all subsequent studies have used PTCy at 50 mg/kg/day on days +3/+4.

This PTCy dosing schema for HLA-haploidentical HCT has shown very encouraging outcomes, with survival rates similar to those observed with HLA-matched HCT using standard GVHD prophylaxis while simultaneously significantly reducing rates of chronic GVHD [10–12]. Yet there has never been subsequent preclinical or clinical assessment performed to determine whether this dosing schema represents the optimal administration of PTCy in HCT. To investigate this question, we assessed the impact of differential dosing and timing of PTCy on its efficacy in reducing GVHD severity in our recently developed MHC-haploidentical murine HCT model [13].

METHODS

Mice

B6C3F1/Crl (donor) and B6D2F1/Crl (recipient) female mice were obtained from the Charles River Laboratories and were 10 to 12 weeks old at HCT. All mice were housed in specific pathogen-free conditions at the National Cancer Institute and were provided food and water ad libitum.

HCT Procedure

Recipient B6D2F1 mice were irradiated to 10.5 Gy in a single dose 6 to 8 hours before HCT via a tail vein injection of 10 × 10^7 T cell-depleted (TCD) B6C3F1 bone marrow (BM) cells or without with or without 40 × 10^6 RBC-Cy7 anti-Epcam (clone 53-6.7), PE-Cy7 anti-H2kd (clone SF1-1.1), and BV605 anti-Ki-67 (clone PC61), and PE H2kk (clone 36-7-5) from BD Biosciences; and PE-Cy5 anti-CD8 (clone 53-5.7) from Bio-X-Cell, West Lebanon, NH), followed by treatment with guinea pig complement (Cedarlane, Burlington, ON, Canada).

Cyclophosphamide Preparation and Treatment

Cyclophosphamide (Baxter Oncology, Halle, Germany) was reconstituted in sterile PBS at 5 or 10 mg/mL, aliquoted, and stored at -80°C. Immediately before treatment, aliquots were thawed and diluted with sterile PBS to .2 mg/mL (for 5 mg/kg dosing), 5 mg/mL (for 10 mg/kg dosing), or 1 mg/mL (for 25 or 50 mg/kg dosing). The dose was determined based on the mouse's weight on the day of each treatment. Cyclophosphamide was administered i.p. Mice not receiving cyclophosphamide on a given day always received similar volumes of sterile PBS i.p.

Flow Cytometry

Blood and spleen collection and processing were done as described previously [13]. In brief, B6C3F1 spleens were mechanically dissociated and RBC-lysed. B6C3F1 BM was flushed out of tibiis and femurs and then T cell-depleted using anti-Thy1.2 antibody (Bio X Cell, West Lebanon, NH), followed by treatment with guinea pig complement (Cedarlane, Burlington, ON, Canada).

RESULTS

PTCy Given on Days +3/+4 Is Superior to Administration on Days +1/2, +5/+6, or +7/+8

To explore the effectiveness of various PTCy dosing strategies, we used our recently described MHC-haploidentical murine HCT model (B6C3F1→B6D2F1) [13]. In this model, PTCy doses of 10 to 50 mg/kg/day on days +3/+4 can effectively prevent fatal GVHD, with 25 mg/kg/day PTCy being the optimal dose, associated with less clinical and histopathological evidence of GVHD than lower or higher doses. A dose of 10 mg/kg/day PTCy on days +3/+4 prevents fatal GVHD in a subset of mice, whereas 5 mg/kg/day on days +3/+4 is ineffective [13].

We began by testing the relative efficacy of both the optimal dose (25 mg/kg/day) and the threshold dose (10 mg/kg/day) when given on days +1/+2 versus +3/+4 versus +5/+6 versus +7/+8. Interestingly, mice treated with 10 mg/kg/day PTCy on days +1/+2 died significantly faster than vehicle-treated mice (hazard ratio [HR], 6.3; P = .0019). In contrast, 10 mg/kg/day PTCy on days +3/+4 or +5/+6 significantly prolonged survival (HR, 0.34; P = .0001 and HR, 0.83, P = .0005; respectively) compared with vehicle-treated mice. PTCy 10 mg/kg/day on days +7/+8 did prolong survival compared with vehicle-treated mice, but this difference was not significant (HR, 4.4; P = .16) (Figure 1A, Table 1). Among the 10 mg/kg/day dosing schedules, PTCy on days +3/+4 was most effective, with 80% of the mice surviving to day +120; this survival was not significantly different than that in the days +5/+6 group (HR, 4.1; P = .19) but was significantly longer than that in the days +7/+8 group (HR, 0.77; P = .0002). These survival differences were mirrored in the weights and clinical scores in which 10 mg/kg/day PTCy given on days +3/+4 was superior not just to vehicle-treated mice, but also to mice given PTCy over the other dosing schedules (Figure 1A, Table 1).

At the optimal PTCy dose (25 mg/kg/day), administration on days +1/+2, +3/+4, +5/+6, or +7/+8 all significantly prolonged survival compared with vehicle treatment (days +1/+2: HR, 13.3; P = .0039; days +3/+4: HR, 0.42, P = .0006; days +5/+6: HR, 0.19, P = .0034; days +7/+8: HR, 0.31, P = .0002), with the days +3/+4 group achieving the highest survival (Figure 1B, Table 1). Mice receiving 25 mg/kg/day PTCy on days +3/+4 had superior weights and clinical scores compared with mice who received vehicle treatment or any of the other three 25 mg/kg/day PTCy dosing schedules (Figure 1B, Table 1).

Statistical Analysis

The exact log-rank test was used to compare survival distributions. The Wilcoxon rank-sum test was used to compare weight and clinical score area under the curve (AUC) values. For AUC analyses, dead mice had their last observation carried forward to subsequent time points. To minimize any bias introduced by using this last observation carried forward approach to impute weight and clinical score values for deceased mice, AUC comparisons were restricted to intervals in which there were ≥7 mice per experimental group. The sole exception was the PTCy dosing on days +1/+2 versus +3/+4 versus +5/+6 versus +7/+8 experiments, in which all mice treated with PTCy 10 mg/kg/day on days +1/+2 died quickly; therefore, results were compared over the interval in which at least 7 of the vehicle-treated mice survived (days 0 through +21). Weight and clinical score data are shown as mean ± SEM. Cell subset percentages were transformed using an arcsin transformation before 1-way analysis of variance (ANOVA). Cell subset absolute numbers or median, or mean fluorescence intensity data were transformed using a natural logarithmic transformation before 1-way ANOVA. When differences were statistically significant, ANOVA was followed by the Holm-Sidak post hoc test. Although transformed data were used for statistical testing, the nontransformed data are displayed for clarity of understanding and are shown as box-and-whisker plots. SAS/STAT version 12 (SAS Institute, Cary, NC) was used for analyses of survival, weight, and clinical score data. Prism version 7.01 (GraphPad Software, La Jolla, CA) was used for statistical analysis of the flow cytometry data and for data presentation. All analyses were 2-tailed. A P value <.05 was considered statistically significant.
Histopathological assessment at day +7 (Figure 2A, Table 1) showed that mice receiving 25 mg/kg/day PTCy on days +3/+4 had only minimal to mild GVHD, which was significantly less severe than mice who received 10 mg/kg/day on days +1/+2 or +3/+4 or 25 mg/kg on days +1/+2 (P < .0001 for all comparisons). Histopathological assessment at day +21 mirrored the clinical results, with the lowest GVHD histopathological severity scores at either dose level corresponding with PTCy administration on days +3/+4 (Figure 2B, Table 1). Overall, these data suggest that PTCy administration on days +3/+4 is superior to administration on days +1/+2, +5/+6, or +7/+8, highlighting the important relationship between the timing of PTCy administration and prevention of GVHD. In addition, the worse outcomes in mice treated with 10 mg/kg/day on days +1/+2 compared with vehicle-treated mice suggests that there may be a biologically significant interaction between the dosing and timing of PTCy.

**PTCy Given on Days +3/+4 Is Superior to Days +2/+3 at Suboptimal Doses**

The optimal day for administration of a single dose of PTCy in the MHC-matched skin allografting models was day +2 or +3, with earlier or later dosing being associated with inferior outcomes [2]. Therefore, we hypothesized that PTCy administration on days +2/+3 might be superior to the standard clinical practice of PTCy dosing on days +3/+4. We compared these 2 dosing schedules using the optimal dose (25 mg/kg/day), the threshold dose (10 mg/kg/day), and also the marginal dose of
The data reported for the clinical scores and weights refer to the comparisons of area under the curve (AUC) analyses over the time points in which there were >7 mice per group as per the figure legends for each set of experiments. The sole exception was for the 10 mg/kg groups in Figure 1, in which all mice treated with 10 mg/kg/day PT Cy on days +1/+2 died rapidly and so the AUC comparisons were over days 0 to 21, after which there were <7 mice in the vehicle-treated group.

<table>
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<tr>
<th>PT Cy Dose</th>
<th>Survival</th>
<th>Clinical Scores</th>
<th>Weights</th>
<th>Histopathology</th>
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<tr>
<td>5 mg/kg</td>
<td>Dosing on days +3/+4 resulted in improved survival compared with dosing on days +2/+3 (not statistically significant; <em>P</em> = .061). No significant differences between dosing on days +3/+4, +4/+5, or +3/+5; none significantly prolonged survival compared with vehicle.</td>
<td>No significant difference between dosing on days +3/+4 or +2/+3. No significant differences between dosing on days +3/+4, +4/+5, or +3/+5. No significant difference between dosing on days +3/+4 or +2/+3.</td>
<td>Not performed</td>
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<td>10 mg/kg</td>
<td>Dosing on days +1/+2 led to accelerated death compared with vehicle (<em>P</em> = .0019). Dosing on days +3/+4 (<em>P</em> &lt; .0001) or +5/+6 (<em>P</em> = .0005) significantly prolonged survival compared with vehicle; dosing on days +7/+8 did not prolong survival. Dosing on days +3/+4 significantly prolonged survival compared with dosing on days +1/+2 (<em>P</em> &lt; .0001) or +7/+8 (<em>P</em> = .0002), but not on days +5/+6 (<em>P</em> = .19). Dosing on days +3/+4 significantly prolonged survival compared with dosing on days +2/+3 (<em>P</em> = .0075). Dosing on days +3/+4 (<em>P</em> = .0054) or +4/+5 (<em>P</em> &lt; .0001) significantly prolonged survival compared with vehicle, but the effect of dosing on days +3/+5 was less (<em>P</em> = .06); no significant differences between these dosing schedules. Dosing on day +4 only (<em>P</em> = .14) or 5 mg/kg/dose b.i.d. on days +3/+4 (<em>P</em> = .058) were only partially effective in prolonging survival compared with vehicle; dosing on day +3 only did not prolong survival.</td>
<td>Dosing on days +3/+4 resulted in significantly better scores compared with vehicle-treated mice (<em>P</em> &lt; .0001) and was superior to dosing on days +1/+2, +5/+6, or +7/+8. Dosing on days +3/+4 resulted in significantly better scores than dosing on days 2/+3 (<em>P</em> = .011). No significant differences between dosing on days +3/+4, +4/+5, or +3/+5; all 3 dosing schedules were significantly better than vehicle (<em>P</em> = .0002, .0001, and .0052, respectively). Dosing on days +3/+4, day +3 only, day +4 only, or 5 mg/kg/dose b.i.d. on days +3/+4 resulted in significantly worse scores than other dosing schedules (<em>P</em> = .0052 versus days +3/+4, <em>P</em> = .034 versus day +4 only, <em>P</em> = .017 versus 5 mg/kg/dose b.i.d. on days +3/+4), but there were no significant differences between the other dosing groups.</td>
<td>Dosing on days +3/+4 resulted in significantly better weights compared with vehicle (<em>P</em> = .023) and was superior to dosing on days +1/+2, +5/+6, or +7/+8. No significant difference between dosing on days +3/+4 or +2/+3. No significant differences between dosing on days +3/+4, +4/+5, or +3/+5; all 3 dosing schedules were significantly better than vehicle (<em>P</em> = .04, .0002, and .004, respectively). No significant differences between dosing on days +3/+4, day +3 only, day +4 only, or 5 mg/kg/dose b.i.d. on days +3/+4.</td>
<td>Day 7: No significant differences compared with vehicle for dosing on days +1/+2 or +3/+4. Day 21: Dosing on days +3/+4 resulted in significantly less GVHD than vehicle (<em>P</em> = .026). No significant differences compared with vehicle for dosing on days +2/+3, +5/+6, or +7/+8.</td>
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<td>25 mg/kg</td>
<td>Dosing on days +1/+2, +3/+4, +5/+6, or +7/+8 all significantly prolonged survival compared with vehicle (<em>P</em> = .0039, .0001, .0084, and .0021, respectively); dosing on days +3/+4 significantly prolonged survival compared with dosing on days +7/+8 (<em>P</em> = .0045), but not on days +1/+2 (<em>P</em> = .24) or +5/+6 (<em>P</em> = .11). No significant difference between dosing on days +3/+4 or +2/+3. No significant differences between dosing on days +3/+4, +4/+5, or +3/+5; all 3 dosing schedules significantly prolonged survival compared with vehicle (<em>P</em> = .0002, .0001, and .0052, respectively). Dosing of 50 mg/kg on day +3 only resulted in significantly worse survival compared with 25 mg/kg on day +4 only (<em>P</em> = .029) or 12.5 mg/kg/dose b.i.d. on days +3/+4 (<em>P</em> = .019); no significant differences between 50 mg/kg on day +3 only and 25 mg/kg/day on days +3/+4 or day +3 only; no significant differences between 25 mg/kg on day +3 only, 25 mg/kg on day +4 only, 25 mg/kg/day on days +3/+4, and 12.5 mg/kg/dose b.i.d. on days +3/+4.</td>
<td>Dosing on days +3/+4 resulted in significantly better scores compared with vehicle (<em>P</em> &lt; .0001) or dosing on days +1/+2, +5/+6, or +7/+8 (<em>P</em> &lt; .0001 for all 3 comparisons). No significant difference between dosing on days +3/+4 or +2/+3. No significant differences between dosing on days +3/+4, +4/+5, or +3/+5. No significant differences between dosing of 12.5 mg/kg/ dose b.i.d. on days +3/+4 or 25 mg/kg/day on day +3 only, day +4 only, or days +3/+4; dosing of 50 mg/kg on day +3 only resulted in significantly worse scores compared with 12.5 mg/kg/dose b.i.d. on days +3/+4 (<em>P</em> = .014).</td>
<td>Dosing on days +3/+4 resulted in superior weights compared with vehicle (<em>P</em> = .0007) or dosing on days +1/+2, +5/+6, or +7/+8 (<em>P</em> = .0011, .052, and .015, respectively). No significant difference between dosing on days +3/+4 or +2/+3. No significant differences between dosing on days +3/+4, +4/+5, or +3/+5. No significant differences between dosing of 50 mg/kg on day +3 only, 12.5 mg/kg/ dose b.i.d. on days +3/+4, or 25 mg/kg/day on days +3/+4, +3 only, or +4 only.</td>
<td>Day 7: Dosing on days +3/+4 resulted in significantly less GVHD compared with vehicle (<em>P</em> = .001), but dosing on days +1/+2 did not result in less histopathological GVHD compared with vehicle. Day 21: Dosing on days +1/+2, +3/+4, +5/+6, or +7/+8 resulted in significantly less GVHD compared with vehicle (<em>P</em> &lt; .0001 for each comparison except <em>P</em> = .0019 for days +7/+8); dosing on days +3/+4 resulted in the least histopathological GVHD.</td>
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PTCy Given on Days +3/+4, +4/+5, or +3/+5 has Similar Efficacy in Preventing GVHD

Given that PTCy was more effective on days +3/+4 than on days +2/+3 (Figure 3) and that dosing on days +5/+6 was partially effective (Figure 1), we next compared PTCy dosing on days +3/+4 versus days +4/+5. Because PTCy also has shown clinical efficacy when given on days +3/+5 [14,15], we also included this dosing schema as a third comparator group. At 5, 10, or 25 mg/kg/day, PTCy was similarly effective on days +3/+4, +4/+5, and +3/+5 (Figure 4, Table 1).

The Efficacy of PTCy Peaks at Day +4, and Both the Dose and Cumulative Exposure Are Critical to its Efficacy

Because the maximal effectiveness of PTCy seemed to center around day +4, we next examined single-day dosing schedules compared with the standard 2-day dosing. In these experiments, we also included experimental groups that would receive a half-dose every 12 hours (b.i.d.) over 2 days to simultaneously examine the relative importance of PTCy cumulative exposure. At lower PTCy doses, only 10 mg/kg/day PTCy on days +3/+4 significantly prolonged survival compared with vehicle treatment (HR, .035; P < .0001), whereas 10 mg/kg PTCy on day +4 only and 5 mg/kg/dose PTCy b.i.d. on days +3/+4 were partially effective in a subset of mice (HR, .45; P = .049 and HR, .38; P = .058, respectively) (Figure 5A, Table 1). In contrast, 10 mg/kg PTCy on day +3 only was completely ineffective at preventing lethal GVHD (P = .64). Consistent with these results, clinical scores were significantly worse in mice treated with 10 mg/kg PTCy on day +3 only compared with the other 3 PTCy treatment groups (10 mg/kg on day +4 only, P = .024; 10 mg/kg/day on days +3/+4, P = .0005; 5 mg/kg/dose b.i.d. on days +3/+4, P = .017). At higher PTCy doses, all dosing schedules significantly prolonged survival compared with vehicle treatment (Figure 5B, Table 1). Mice receiving 25 mg/kg PTCy on day +4 only had the highest survival rate, followed by those receiving 12.5 mg/kg/dose b.i.d. on days +3/+4, 25 mg/kg/day on days +3/+4, and 25 mg/kg on day +3 only; yet none of these were significantly different from one another (P ≥ .21 for all). However, mice treated with 50 mg/kg PTCy on day +3 only had significantly worse survival than those treated with 25 mg/kg PTCy on day +4 only (HR, 0; P = .029) or 12.5 mg/kg/dose PTCy b.i.d. on days +3/+4 (HR, 12; P = .019), but not significantly worse than those treated with 25 mg/kg/day PTCy on days +3/+4 (HR, .15; P = .801) or 25 mg/kg PTCy on day +3 only (HR, .49; P = .38). There were no differences in weights between PTCy dosing schedules. The 50 mg/kg PTCy on day +3 only group had worse clinical scores than the 12.5 mg/kg/dose PTCy b.i.d. on days +3/+4 group (P = .014), but otherwise there were no significant differences between groups. Overall, these data suggest that PTCy reaches its maximal efficacy when given on day +4, and that PTCy given on day +4 only may be as effective as PTCy.
given on days +3/+4 at the optimal dose. These results also highlight the importance of both the timing and the cumulative exposure of PTCy for its effectiveness.

**Effective PTCy Dosing Schemas Reduce Alloreactive CD4+ Conventional T Cell Proliferation at Day +7**

We recently demonstrated that PTCy does not selectively eliminate alloreactive T cells [13]. Over the course of that work, we found that 25 mg/kg/day PTCy on days +3/+4 both reduced CD4+ T cell proliferation at day +7 and resulted in preferential CD4+CD25+Foxp3+ regulatory T cell (Treg) recovery at day +21 [13]. PTCy 100 mg/kg/day on days +3/+4, which was less effective at GVHD prevention than 25 mg/kg/day, did decrease CD4+ T cell proliferation at day +7 but did not result in preferential Treg recovery at day +21, whereas the ineffective low dose of 5 mg/kg/day PTCy on days +3/+4 did not achieve either goal [13]. Therefore, to gain biological insight into why certain dosing schema are more effective than others, we performed flow cytometric analysis at days +7 and +21 after various PTCy dosing schedules. We hypothesized that these 2 parameters may be potential biomarkers of effective GVHD prevention by PTCy.
In previous work, we looked at 5 different markers of alloreactive T cells [13]. Of these, the only one applicable to the wild-type mice used in the B6C3F1 × B6D2F1 HCT model is Vβ6 [16]. We found that Vβ6⁺ CD4⁺CD25⁺Foxp3⁺ conventional T cells (T_{cons}) and Vβ6⁺ CD8⁺ T cells persisted at day +7 at similar to higher percentages in all PTCy-treated mice compared with vehicle-treated mice, although total numbers were reduced for some PTCy-treated groups (Figure 6A and Supplementary Figure S1); these percentages also were similar at day +21 (Figure 6B). However, the proliferation of

Figure 4. PTCy has similar efficacy when given on days +3/+4, +4/+5, or +3/+5. Mice underwent transplantation as in Figure 1 and received PTCy at 5 mg/kg/day (A), 10 mg/kg/day (B), or 25 mg/kg/day (C) on designated days. Mice not receiving PTCy on a given day received PBS vehicle. The same TCD BM, vehicle and TCD BM, Splen, Vehicle control groups are shown in all parts for comparison purposes. Statistical comparisons were performed between PTCy treatment groups and the TCD BM, Splen, Vehicle control group and between different PTCy treatment groups. (A) There were no significant differences in survival, weight AUCs (days 0 to 15), or clinical score AUCs (days 0 to 15) between the different 5 mg/kg/day PTCy dosing schedules, and none significantly prolonged survival compared with vehicle-treated mice. (B) 10 mg/kg/day PTCy on either days +3/+4 (HR, 2; P = .0054) or days +4/+5 (HR, 1; P = .0001) significantly prolonged survival compared with vehicle-treated mice, whereas the impact on survival of PTCy on days +3/+5 was less (HR, 3; P = .06). Although survival was best after 10 mg/kg/day PTCy on days +4/+5, there were no significant differences between treatment groups (days +4/+5 compared with days +3/+5, P = .07). Weight AUCs (days 0 to 18) were similar between treatment groups, and significantly better in all treatment groups than in vehicle-treated mice. On pointwise comparison, days +4/+5 had higher weights compared with days +3/+4 on day +18 (P = .02) and compared with days +3/+5 on days +15 and +18 (P < .0003 for both days). Clinical score AUCs were similar between 10 mg/kg/day PTCy treatment groups, and all 3 treatment groups were significantly better than vehicle-treated mice (days +3/+4, P = .0002; days +4/+5, P = .0001; days +3/+5, P = .0052). (C) At the 25 mg/kg/day dosing, there were no differences in survival across the treatment groups, but all 3 treatment groups had significantly prolonged survival compared with the vehicle-treated group (days +3/+4: HR, 0.6, P = .0002; day +4/+5: HR, 0.3, P = .0001; days +3/+5: HR, 0.6, P = .0002). Weight and clinical score AUCs over the entire period were similar among the three 25 mg/kg/day PTCy dosing schedules. Combined results are shown for 2 independent experiments of 5 mice/group/experiment.
Vβ6+ CD4+CD25+ Foxp3+ T_con at day +7 was affected by PTCy. PTCy given at either 10 mg/kg/day or 25 mg/kg/day on days +3/+4 or +5/+6 significantly reduced Vβ6+ CD4+CD25+ Foxp3+ T_con proliferation at day +7 in the spleen, whereas only 25 mg/kg/day PTCy on days +3/+4 significantly reduced Vβ6+ CD4+CD25+ Foxp3+ T_con proliferation in the blood (Figure 6C and Supplementary Figure S2). Interestingly, Vβ6+ CD4+CD25+ Foxp3+ T_con proliferation was significantly increased in mice treated with 10 mg/kg/day PTCy on days +1/+2 (Figure 6C and Supplementary Figure S2), a dose associated with more rapid lethality from GVHD, compared with in vehicle-treated mice (Figure 1A).
Effective PTCy dosing schemas facilitate preferential T\textsubscript{reg} reconstitution by day +21

T\textsubscript{regs} have been shown to play a necessary role in GVHD prevention by PTCy in xenogeneic [17], MHC-matched [18], and MHC-haploidentical [13] murine HCT models. In our MHC-haploidentical model, at day +7 there were similar percentages of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} T\textsubscript{regs} in blood and lymph nodes and slightly lower percentages in spleen and liver in PTCy-treated mice compared with vehicle-treated mice, but at day +21 after 25 mg/kg/day PTCy on days +3/+4, the percentages of T\textsubscript{regs} had become significantly increased in all 4 organs [13].

Consistent with our previous results [13], at day +7 we found no differences in T\textsubscript{reg} percentages in blood irrespective of PTCy dosing schedule, whereas the T\textsubscript{reg} percentages in spleen were lower after most PTCy dosing schedules (Figure 7A). Moreover, at
day +7, we found no significant differences in the percentages of alloantigen-specific (V\(\beta\)6\(^+\)) T\(_{\text{regs}}\) (Figure 7B).

Supportive of our hypothesis, we found that all the most effective PTCy dosing schedules (10 mg/kg/day on days +3/+4 and 25 mg/kg/day on days +1/+2, +3/+4, or +5/+6) resulted in significantly increased percentages of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T\(_{\text{regs}}\) at day +21 in both blood and spleen, with maximal increases in these percentages after 25 mg/kg/day PTCy on days +1/+2 or

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**Figure 7.** Effective dosing schedules of PTCy result in preferential recovery of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T\(_{\text{regs}}\) at day +21. Mice underwent transplantation as in Figure 1 and received PTCy at various dosing schedules. Mice not receiving PTCy on a given day received PBS vehicle. At designated time points, mice were euthanized, and their spleens and blood were assessed by flow cytometry. (A) At day +7, the percentages of CD4\(^+\) T cells that were CD25\(^+\)Foxp3\(^+\) were similar or reduced by various PTCy dosing schedules compared with vehicle treatment. (B) The percentages of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T\(_{\text{regs}}\) that were V\(\beta\)6\(^+\) were not significantly different between groups at day +7. (C) At day +21, the most effective doses of PTCy (10 mg/kg/day on days +3/+4 or 25 mg/kg/day on days +1/+2, +3/+4, or +5/+6) were associated with significantly increased percentages of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T\(_{\text{regs}}\) in both blood and spleen. (D) The percentages of V\(\beta\)6\(^+\) T\(_{\text{regs}}\) were not significantly different at day +21 among the treatment groups, although the more effective dosing schedules tended to have higher percentages. (E and F) Pooling across all treatment groups, the percentages of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T\(_{\text{regs}}\) in either blood (E) or spleen (F) were significantly negatively associated with the histopathological severity scores in the same mice, suggesting that the percentage of T\(_{\text{regs}}\) at day +21 in this model may be a biomarker of effective GVHD prevention. Combined results from 2 independent experiments are shown. N = 6/group for (A) and (B) except the 10 mg/kg/day or 25 mg/kg/day PTCy on days +1/+2 groups (n = 4 each) and the 25 mg/kg/day PTCy on days +5/+6 group (n = 5) because of some deaths occurring before day +7. N = 8/group for (C) to (F) except 10 mg/kg/day PTCy on days +2/+3 (n = 4). *P ≤ .05; **P ≤ .01; ***P ≤ .001; ****P ≤ .0001, 1-way ANOVA followed by the Holm-Sidak post hoc test using the vehicle-treated group as the control. Only significant results are shown; all other comparisons between the vehicle treatment group and each PTCy treatment group are nonsignificant.
+3/+4 (Figure 7C). Given the limited power, there were no significant differences in the percentage of Vα6Treg, although the highest percentages were seen in the mice receiving 25 mg/kg/day PTCy on days +3/+4 (Figure 7D). Absolute numbers of total CD4+CD25+Foxp3+ Treg and Vα6 Treg at day +21 were similar or significantly increased in PTCy-treated mice compared with vehicle-treated mice (Supplementary Figure S3). The percentages of all Treg in the blood or spleen at day +21 correlated negatively with the GVHD histopathological severity score within the same mice (Figure 7E and F). These data suggest that both the initial reduction of alloreactive CD4+ Tcon proliferation at day +7 and subsequent Treg reconstitution at day +21 may be potential biomarkers of effective GVHD prevention by PTCy.

**DISCUSSION**

In this work, we used a murine MHC-haploidentical HCT model to evaluate the impact of the timing of PTCy on GVHD prevention. We found that the efficacy of PTCy peaked at day +4, although the window for effective GVHD prophylaxis was broader at the optimal dose. Moreover, our findings highlight the importance of the dose and timing of PTCy, but also the cumulative exposure of the drug for effective prevention of GVHD, as demonstrated by the b.i.d. and the single-day versus 2-day dosing schedules.

Herein, we also found that the optimal PTCy dosing schema both reduced alloreactive CD4+ Tcon proliferation at day +7 and facilitated preferential Treg recovery at day +21, adding potential biologic insight into why certain dosing schemas are optimal. These results are consistent with our previous findings that too high a PTCy dose may be ineffective because it blocks the preferential recovery of Treg compared with Tcon despite much more effective reduction of alloreactive CD4+ Tcon proliferation at day +7 [13]. In the present study, PTCy on days +1/+2 allowed Treg recovery at day +21 similar to that observed after PTCy on days +3/+4, but the days +1/+2 dose schedule did not adequately abate alloreactive CD4+ Tcon proliferation at day +7. In contrast, PTCy on days +5/+6 substantially reduced alloreactive CD4+ Tcon proliferation at day +7 but yielded much less robust Treg recovery at day +21. Moreover, 10 mg/kg/day PTCy on days +1/+2 actually increased alloreactive CD4+ Tcon proliferation at day +7, leading to rapid death in these mice. Although it is unclear whether these immunologic parameters are mechanistic or simply epiphenomena, Tregs are necessary for GVHD prevention by PTCy [13,17,18], and at the very least, this combination appears to be a potential predictive biomarker for effective GVHD prevention by PTCy.

Further study is needed to understand the extent to which these findings apply to human HCT. An important caveat is that we do not know how cyclophosphamide doses correspond between mice and humans, particularly considering the pharmacokinetic differences, with mice having quicker clearance of the drug [19]. Our previous study suggested that an intermediate PTCy dose (25 mg/kg/day) was optimal in 2 different murine HCT models, with both lower and higher doses associated with increased GVHD and mortality [13]. In humans, the current clinical dose of 50 mg/kg/day is nearly as high a cyclophosphamide dose as a human can tolerate. PTCy 25 mg/kg/day on days +3/+4 has shown efficacy in small clinical studies [20,21], as has 14.5 mg/kg/day in combination with anthymocyte globulin [22]. Even PTCy at 7.5 mg/kg/day on serial dosing between days +1 and +100 has demonstrated efficacy in reducing chronic GVHD [23]; this treatment had minimal impact on acute GVHD, consistent with the results of our mice treated with 10 mg/kg/day, which showed considerable histopathological and clinical GVHD early and slow improvement over 4 months. Thus, the clinical observations in mice and humans suggest that doses may have relatively similar effects in the 2 species.

Our work has several other limitations. First, our murine studies were underpowered to detect small differences between dosing schemas. Even so, we identified that the optimal window centered around day +4. Previous clinical studies have suggested that dosing on days +3/+4 may be associated with lower rates of GVHD than dosing on day +3 alone [9,21], and that PTCy on days +3/+5 is quite effective [15]. The latter approach results in very low rates of acute GVHD as well, but that strategy also begins the adjunct immunosuppression on the day of HCT instead of on day +5. This difference highlights another limitation of the translatability of results from our murine HCT model in which PTCy is used alone for GVHD prevention. The adjunct prophylactic immunosuppression generally given in addition to PTCy in patients impacts clinically on rates of acute GVHD and biologically on alloreactive T cell proliferation and also may impact Treg recovery [24]. Thus, these other agents may obscure the maximal efficacy of PTCy itself on clinical and immunologic parameters and potentially could impact which dosing schemas are optimal in specific clinical contexts. In addition, there may be differences between mouse and human T cells in the kinetics of proliferation and expression of cyclophosphamide resistance pathways, such as aldehyde dehydrogenase [17,18]. It is also unknown whether the effects on Vα6 T cells found here reflect changes in other alloreactive T cells in mice or humans; however, our recent work did show similar effects of PTCy on other types of alloreactive T cells as PTCy did on Vα6 T cells [13], but human data are needed to determine whether these results apply to clinical HCT. Furthermore, Ki-67 is a marker of T cells in active cell cycle and consequently is only an indirect marker of cell division. Even so, this marker is easily testable in human HCT, and whether cell division is more important than proliferation (cell cycle) in this context is unclear. Finally, our B6C3F1→B6D2F1 MHC-haploidentical HCT model is primarily one of acute rather than chronic GVHD, and optimal dosing schemas of PTCy possibly could differ for prevention of each.

Our findings contrast with the results of skin allografting studies that determined that PTCy most effectively promoted engraftment when given on day +2 or +3 and when using a very high dose (200 mg/kg) [2]. However, PTCy’s efficacy in those studies was contextual, requiring specific type and dose of donor cells and MHC-matched recipients of a specific age [2,25,26]. Moreover, our recent work has highlighted important mechanistic differences between PTCy’s activity in preventing GVHD after HCT compared with what has been previously described for preventing MHC-matched skin allograft rejection, possibly contributing to the differing optimal dosing schedules [13]. Furthermore, a high dose of PTCy at an earlier time point potentially may overcome the less robust impact on alloreactive CD4+ Tcon proliferation of lower doses of PTCy given on days +1/+2 or +2/+3 while taking advantage of the similar preferential Treg recovery occurring at that time point, thereby rebalancing the net impact of different PTCy dosing schemas; such a theorized effect may be more active in a mixed chimeric state when trying to induce host tolerance to a subsequently placed skin allograft rather than controlling active graft-versus-host alloreactivity.

In conclusion, our findings highlight the importance of the dose, timing, and cumulative exposure of PTCy on its clinical effectiveness in preventing GVHD. Our findings support that the current timing of PTCy administration may be among the best
options, but suggest that when given at the optimal dose, PTCy may have equal efficacy when given on day +4 alone. These data support a clinical study initiated at our institution to explore PTCy dose de-escalation first to 25 mg/kg/day on days +3/+4 and then to 25 mg/kg on day +4 only. If successful, this strategy may reduce toxicity and improve hematopoietic and immune reconstitution while maintaining severe acute and chronic GVHD prevention. In this clinical study, we simultaneously are evaluating candidate predictive immunologic biomarkers for effective GVHD prevention by PTCy of CD4+ T cell proliferation early after PTCy and CD4+CD25+Foxp3+ recovery at day +21 in a quantitative systems pharmacology model. We hope that these studies will improve clinical HCT outcomes for patients and provide further insight into the biology of acute and chronic GVHD and their prevention.

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SUPPLEMENTARY MATERIALS
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