Antigen and Lymphopenia-Driven Donor T Cells Are Differentially Diminished by Post-Transplantation Administration of Cyclophosphamide after Hematopoietic Cell Transplantation

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ABSTRACT
Administration of cyclophosphamide after transplantation (post-transplantation cyclophosphamide, PTC) has shown promise in the clinic as a prophylactic agent against graft-versus-host disease (GVHD). An important issue with regard to recipient immune function and reconstitution after PTC is the extent to which, in addition to diminution of antihost allo-reactive donor T cells, the remainder of the nonhost allo-reactive donor T cell pool may be affected. To investigate PTC's effects on nonhost reactive donor CD8 T cells, ova-specific (OT-I) and gp100-specific Pmel-1 T cells were labeled with proliferation dyes and transplanted into syngeneic and allogeneic recipients. Notably, an intermediate dose (66 mg/kg) of PTC, which abrogated GVHD after allogeneic HSCT, did not significantly diminish these peptide-specific donor T cell populations. Analysis of the rate of proliferation after transplantation illustrated that lymphopenic-driven, donor nonhost reactive TCR Tg T cells in syngeneic recipients underwent slow division, resulting in significant sparing of these donor populations. In contrast, after exposure to specific antigens at the time of transplantation, these same T cells were significantly depleted by PTC, demonstrating the global susceptibility of rapidly dividing T cells after an encounter with cognate antigen. In total, our results, employing both syngeneic and allogeneic minor antigen-mismatched T cell replete models of transplantation, demonstrate a concentration of PTC that significantly depleted by PTC, demonstrating the global susceptibility of rapidly dividing T cells after an encounter with cognate antigen.

INTRODUCTION
Allogeneic hematopoietic stem cell transplantation (AH SCT) is a curative therapy for some blood cancers and has the potential to be applied to many other malignancies, although such use is hindered by the complication of graft-versus-host disease (GVHD) [1-5]. Graft-versus-host responses are immediately initiated after transplantation by rapidly cycling donor T cells that are not tolerant to host allogeneic transplantation antigens [6-10]. Efforts to remove antihost alloantigen reactive T cells ex vivo before transplantation are ongoing, but practical as well as technical issues have thus far precluded development of an effective strategy [7,11,12]. Additionally, the low frequency of T cells reactive with non-HLA-encoded (ie, minor) transplantation antigens provides added challenges for successful ex vivo deletion strategies [13,14]. Alkylating compounds induce breaks in DNA, which initiate the apoptosis of the affected cells upon entry into the replication cycle or necrotic death, dependent on the cell population and conditions present [15,16]. Regardless, these agents principally target dividing cells. Studies utilizing alkylating agents in attempts to impart immune tolerance were initiated in the late 1950s in preclinical models [17-19]. Early studies demonstrated that cyclophosphamide, an alkylating agent, could diminish donor antihost reactive T cells after an allogeneic tissue graft [20]. Subsequent work found that after low-dose total body irradiation (TBI) conditioning and allogeneic bone marrow infusion, cyclophosphamide administration could prevent host T cells, which were responding to donor antigens, from rejecting the graft and enabled donor hematopoietic engraftment [21].

These findings, in part, rekindled interest in cyclophosphamide as a transient immunosuppressive strategy for patients receiving AH SCT [22]. Recently, clinical trials have been performed at several centers to begin assessing the efficacy of post-transplantation cyclophosphamide (PTC) administration to ameliorate GVHD (http://clinicaltrials.gov/show/NCT01427881) [23-25]. Results are thus far promising for both safety and efficacy of high-dose PTC administration, as well as GVHD occurrence after both nonmyeloablative and myeloablative conditioning in HLA-mismatched and HLA-matched allogeneic HSCT recipients [26-28]. Depending on the extent of conditioning and the status of the patient, T cell–replete AH SCT is performed in the context of varying degrees of lympho-depletion in the recipient. This post-transplantation environment therefore supports both lymphopenia-induced proliferation (LIP) antigen as well as recipient allo-antigen antigen-stimulated proliferation, the former driven by an excess of cytokines present that support T cell homeostasis and maintenance in lympho-replete immune compartments (eg, IL-7, IL-15) [29-32]. Because a major challenge after HSCT is reconstituting immune function as quickly as possible [33-38], a critical question after exposure to PTC concerns what populations of

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donor T cells are diminished or eliminated in recipients. Notably, pretransplantation conditioning was not employed in the historical allograft experiments, and in the preclinical studies that examined engraftment, immune function was not examined. Questions, therefore, remain regarding the susceptibility of T cells undergoing LIP to deletion after PTC administration. The goal of the current study was to examine populations of T cells dividing because of lymphopenia alone or together with antigen-driven activation in response to host allo-antigen or specific peptide antigen in hematopoietic stem cell transplantation models after exposure to PTC. The results demonstrated that PTC has a markedly different impact on host reactive compared to nonhost reactive transplanted donor T cells—the latter were minimally affected by doses of PTC that ameliorated GVHD. These findings are discussed in the context of potential benefits of PTC to facilitate immune responsiveness and reconstitution after AHSCCT.

MATERIALS AND METHODS

Mice

Seven to 8-week-old female C57BL/6 (B6), BALB/c, C3H.SW, and BALB.B (C57BL-H2 b/LimJcd) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in pathogen-free conditions in the Department of Microbiology and Immunology at the University of Miami Miller School of Medicine. B6 mice congenic for CD45 and expressing the CD45.1 allele (-6.5J-PrPcr/BoaTae), Pten-1(-6.5Cg-Thy-1a/Cg Tg (Tcrz2fsfr) R8esfz)])Rac-/- mice [Pten-1 were a gift from Dr. Claudia Marcela Diaz, and OT-1(-C57BL/6-Tg (Tcrzfsfr)1100Mjb/J)]Rag-/- mice were bred in facility.

Bone Marrow Transplantation

Donor C57BL/6 bone marrow, lymph nodes, and spleen were aseptically removed. Single-cell suspensions of marrow, spleen, and lymph node cells were washed in PBS. Donor bone marrow cells were treated with anti-Thy-1.2 MACs magnetic beads (Miltenyi Biotech Inc. Auburn, CA) and negatively selected to remove T cells and washed before transplantation. Spleen and lymph node cells were incubated on anti-Fcγ-coated (Millipore (Billerica, MA)) plastic dishes for 45 minutes at 4°C to remove B cells. Nonadherent cells were harvested and a small aliquot was stained with anti-CD4 and anti-CD8 mAbs to determine percentage contributions. Recipient C3H.SW mice were irradiated with 10.5 Gy gamma-irradiation from an open beam cobalt-60 source (Gamma beam 150). Within 4 hours after irradiation, experimental C3H.SW recipients were injected with C57BL/6 (45.1-1) allogeneic bone marrow plus 2.3 × 106 slg-depleted CD4+ and CD8+ allogeneic T cells (i.v. in 5 mL PBS). Transgenic donor CDB T cell populations administrated were enriched using Miltenyi MACs magnetic bead separation and infused at >90% purity. Recipient BALB.B mice were irradiated with 7.5 Gy gamma-irradiation from an open beam cobalt-60 source (Gamma beam 150). CD8 T cells—depleted bone marrow (TCDBM) transplant recipients were injected with allogeneic bone marrow (i.v. in 5 mL PBS). In recipients of post-transplantation cyclophosphamide (Sigma St. Louis, MO), the indicated concentrations were administered intraperitoneally on days 3 or days 3 and 4 and adjusted for weight of recipients.

Immunofluorescence Staining and Analysis

The following fluorescent antibodies were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), Biologend, and Invitrogen/Life-Technologies—Molecular Probes (Carlsbad, CA) and used for flow cytometric analysis: anti-vv2 (B20.1), anti-vv5/15.2 (BB9.4), anti-CD90.1 (OX-7), anti-CD42 I (MEI-14), anti-CD8a (53-6.7), anti-CD4 (RM4-5), anti-CD19 (6D5), anti-CD45.1(A20), anti-CD44 (IM7), and anti-IFN-γ (XMG1.2). For intracellular cytokine staining, single-cell suspensions prepared from tissues were incubated (1 × 106/mL) in 100% complete medium with 1 nM OVA257-264 (SIINFEKL) or 1 nM human gp10025-33, KVPRNQDWL (kindly provided by Dr. Marcela Diaz-Montero, Department of Medicine, University of Miami School of Medicine) and 2 μg BD GolgiStop protein transport inhibitor containing monensin for 4 to 18 hours at 37°C, surface stained, fixed and permeabilized with the FoxP3 staining kit (eBioscience) overnight, stained intracellularly with the appropriate antibodies, washed, and analyzed.

GVHD Assessment

Recipient mice were monitored for changes in total body weight and overall survival. The clinical signs of GVHD were recorded for individual mice by a marked difference in host reactive compared to nonhost reactive transplanted donor T cells—the latter were minimally affected by doses of PTC that ameliorated GVHD. These findings are discussed in the context of potential benefits of PTC to facilitate immune responsiveness and reconstitution after AHSCCT.

RESULTS

Identification of a PTC Dose That Ameliorated GVHD

To identify an effective dose of PTC that would suppress donor antithost alloreactions leading to GVHD, we employed an allogeneic minor antigen—mismatched hematopoietic stem cell transplantation model of GVHD involving an MHC (H2I)-matched donor and recipient strain combination B6→C3H.SW [40,41]. Groups of C3H.SW recipients exposed to 10.5 Gy TBI (Figure S1A) were injected with donor B6 TCD BM alone or together with 2.3 × 106 B6 T cells. Varying doses of PTC recipients (data not shown) were examined in C3H.SW recipients over the course of several experiments (11 mg/kg to 66 mg/kg per dose delivered in 1 or 2 doses, which represents 275 mg to 3.3 mg total dose per 25 g mice), and single-dose 200 mg/kg, which equaled 5.0 mgs per 25 g mice, and was lethal in C3H.SW recipients (data not shown). In this AHSCCT model, a dose of 33 mg/kg but not 11 mg/kg administered on days 3 and 4 was found to significantly ameliorate GVHD-associated weight loss induced by infusion of 2.3 × 106 slg-depleted B6 splenocytes when C3H.SW recipients were conditioned with lethal (10.5 Gy) TBI (Figure 1A). Notably, improvement in survival (Figure S1) and GVHD clinical score (data not shown) were also observed when 33 mg/kg × 2 (66 mg total) was employed.

To more closely examine the effects of PTC on dividing donor cell populations, slg-depleted B6 splenocytes labeled with a carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation dye and transplanted into C3H.SW recipients. We then examined the effects of 2 doses of PTC, 2 × 11 mg/kg and 2 × 33 mg/kg, on dividing donor CD8+ T cells 5 days after transplantation. PTC administration caused depletion of peaks representing later generations of rapidly dividing cells in a dose-dependent fashion. Notably,
at a concentration of PTC that was unable to abrogate GVHD (2/C211 mg/kg), a population of cells remained evident that divided greater than 5 times in the 5-day experimental period. In contrast, at the 2/C233 mg/kg concentration, which successfully diminished GVHD, the profile was distinctly different in that: (1) there was a more apparent diminution of cells in later (>2) generations, and (2) only 2 to 3 generations of early dividing cells remained (Figure 1B). In total,

Figure 1. PTC after MHC-matched allogeneic HSCT ameliorates GVHD and reduces the percentage of donor T cells in later divisions 5 days after transplantation. (A) GVHD-induced weight loss was reduced after 2 × 33 mg/kg PTC (○, n = 6) and was not significantly different from recipients of TCD-BM only (●, [P > .05; n = 5]). Recipients of 11 mg/kg PTC (△, n = 5) exhibited slightly decreased weight loss versus the non–PTC-treated recipients (□, n = 7) but did not differ significantly (P > .5). A representative experiment of 3 is shown. (B) To analyze how PTC impacts donor T cells early after HSCT, B6 sIg-depleted CD4 and CD8 T cells were labeled with CFSE and transferred into lethal TBI (10.5 Gy)-conditioned C3HSW recipients (n = 5 per group). Varying doses (11 mg/kg or 33 mg/kg) of cyclophosphamide (PTC) were administered i.p. at days 3 and 4 after transplantation. Data represents the percentage of donor T cells that had undergone at least 2 divisions at days 4 to 5 after transplantation. The CFSE dilution profiles indicated that there was greater reduction within the later generations of rapidly dividing donor CD8 T cells.

Figure 2. PTC significantly reduces transplanted donor T cell numbers after allogeneic but not syngeneic HSCT. (A) Cells responding to lymphopenia-induced proliferation are susceptible to PTC in a dose-dependent manner. Note that the loss is most apparent in the more rapidly dividing generations. B6-CD45.1 or C3H.SW splg-CD4 and CD8 T cells were labeled with CFSE and PKH (when required) and injected into lethally conditioned (10.5 Gy) congenic and syngeneic recipients. Results presented as representative histograms from individual mice (B6, n = 6; C3H.SW, n = 4) (B) Total cell numbers present in spleens of recipients 5 to 7 days postsyngeneic (upper panel) or allogeneic (lower panel) transplantation. Three strains were utilized for the syngeneic transplantations: B6, C3H.SW, and BALB.B. Allogeneic transplantations were performed using B6 → BALB.B. Recipients received 2 doses of 33 mg/kg PTC on days 3 and 4 after transplantation. Results represent the composite from 7 and 5 independent syngeneic and allogeneic experiments, respectively. No significant decrease in cell numbers was observed in syngeneic recipients after administration of PTC. In contrast, cell numbers were significantly reduced in allogeneic recipients after treatment with the same PTC dose.
PTC diminished the percentage of proliferating donor T cells in a dose-dependent fashion; however, some slowly dividing (1 to 2 divisions) cells were not deleted even after the 2/C2 33 mg/kg protocol. The loss of rapidly dividing cells after this PTC exposure was observed in many experiments using several distinct strain combinations (data not shown).

**Donor Cells Dividing Because of Lymphopenia Are Essentially Spared after Exposure to PTC**

Previous experiments by ourselves (D.R., R.B.L., unpublished observations) and others have shown that cells dividing because of lymphopenia divide approximately once per 24 hours [42-44]. To elucidate whether 2 times the injection of 33 mg/kg PTC spared the cells that were dividing solely because of lymphopenia, CFSE-labeled T cells were infused into lethally conditioned syngeneic recipients. At a concentration of PTC that ameliorated GVHD after AHSCT in the allogeneic transplantation (2 × 33 mg/kg), the effect of PTC in both B6 and C3H.SW recipients of syngeneic sIg-depleted spleen cells was minimal to cells that had divided more than once, with more than 50% of cells remaining after treatment. A more vigorous depletion of cells was observed at a higher concentration of PTC (ie, 2/C2 66 mg/kg); however, (1) T cells that had not undergone division were again present, and (2) some T cells that had undergone only 1 or 2 divisions were again observed (Figure 2A).

The numbers of donor cells present after PTC was then determined in syngeneic and allogeneic transplant recipients (Figure 2B). No significant diminution in total cell numbers was observed in syngeneic recipients examined 5 to 7 days...
after transplantation after administration of 33 mg/kg on days 3 and 4 (Figure 2B, top). In contrast, a significant loss of cell number was observed in the spleens of B6→BALB.B allologic transplantation experiments after the identical day 3 and 4 PTC regimen after transplantation (Figure 2B, bottom). These findings were consistent with the CFSE dilution patterns observed after syngeneic and allologic transplantation.

Based on the observations that donor CD8+ T cells dividing as a consequence of lymphopenia were diminished to a small degree, by PTC administered at a concentration (33 mg/kg × 2) that diminished GVHD (Figure 1), antigen-specific T cells were subsequently included in the transplantation inoculum to enable more precise monitoring of the impact of the PTC regimen on nonantigen-driven donor T cell proliferation after transplantation. Purified CD8+ populations were obtained from B6-OT-I/Rag−/− and B6-Pmel-1/Rag−/− TcR transgenic mice, labeled with cell proliferation dye, and coadministered with B6 T cells to lethally ablated syngeneic B6 recipients. We observed, as reported by others, after sublethal irradiation [31], the rate of LIP by the CD8 OT-I population (~1/day) was significantly more rapid than that by the Pmel-1 (~1 to 2 divisions through week 1 after transplantation) CD8 T cells after transplantation into syngeneic, lethally irradiated mice (Figure 3A). After 2 × 33 mg/kg PTC administration, minimal diminution of CD8+ Pmel-1 T cells was observed and a small but consistent decrease was detected in the OT-I population (Figure 3A). This effect on the OT-I population was further evidenced in several experiments in which examination of OT-I T cells identified a decrease—although just at the margin of statistical significance (P = .047)—in the percentage of these CD8 T cells in the second or greater division after PTC was administered on days 3 and 4 after transplantation in syngeneic recipients (85.6% versus 77.9% with and without PTC, respectively, Figure 3B, upper). A similar pattern was also observed after coinfused polyclonal B6 CD8+ T cells were transplanted and analyzed in syngeneic recipients (ie, no statistically significant decrease was observed whether or not 2 × 33 mg/kg PTC was administered) (Figure 3B, lower).

We next asked whether cells that had been exposed to PTC and persisted in transplant recipients maintained functional responsiveness when stimulated by cognate antigen. Syngeneic transplantations were, therefore, performed with coadministered CD8+ OT-I T cells, B6 T cells (sIg-depleted syngeneic splenocytes), and 5 × 10^6 B6 (CD45.1) TCD-BM. Six to 8 weeks after transplantation, recipients who were untreated or treated with PTC were exposed to ovalbumin antigen after injection of splenocytes (2 × 10^7) from B6-OVA-transgenic. The presence and expansion (see below) of the transplanted donor OT-I (Vα2-Vβ5+CD8+ T cells) were evident in the blood of both untreated and PTC-treated recipient groups (Figure 3C). Additionally, splenocytes obtained after B6-ova (antigen) injection from these transplantation groups of recipients were stimulated in vitro with ova-peptide (SIINFEKL) for 6 hours and assessed for IFNγ production. Strong intracellular staining with anti-IFNγ mAb was observed in donor B6-CD8+ Vα2+ T cells stimulated ex vivo with SIINFEKL—but not an unrelated nonameric class I H-2 Kb binding peptide (Figure 3D).

To more precisely quantify persistence and expansion of OT-I T cells after 2 × 33 mg/kg PTC administration, syngeneic B6 mice transplanted with 2.3 × 10^6 polyclonal T cells and 1 × 10^6 CD8-OT-I cells were sacrificed and numbers of CD8−Vα2−Vβ8+ positive donor B6 T cells in the spleen were calculated. Two months after transplantation, the number of
OT-I T cells present was not significantly different in groups that received D.3 and 4 PTC administration compared with the group that did not receive PTC (Figure 4A). To determine the susceptibility of B6 CD8^+ OT-I T cells to this PTC regimen under antigen–driven proliferation, these transgenic nonhost reactive CD8 T cells together with B6 T cells were transplanted into syngeneic recipients and specific antigen introduced (day 0) by injection of 2 x 10^7 splenocytes from B6-ova Tg mice (Figure 4B). In contrast to findings obtained in the absence of ovalbumin antigen (Figure 3), OT-I T cells were significantly depleted after days 3 and 4 (2 x 33 mg/kg) of PTC treatment (Figure 4B). These findings demonstrated the effectiveness of the 2 x 33 mg/kg dose to deplete any donor T cell population after efficient antigen stimulation at the time of transplantation before PTC exposure.

In contrast to the 2 PTC dose injection regimen used in clinical transplantations with a matched unrelated donor, haplo-identical HSCT is currently performed using a single injection of PTC [26]. To assess if a single PTC dose containing the same total amount of cyclophosphamide resulted in similar persistence of nonhost reactive donor CD8 T cells, B6-CD45.2^+ OT-I cells were coinfused with B6 CD45.1^+ T cells and bone marrow cells into lethally conditioned C3H.SW CD45.2^+ recipients, followed by injection of 66 mg/kg PTC at day 3 after transplantation (Figure 5). Recipients of donor T cells with or without coinfused B6 OT-I T cells who were treated with PTC did not exhibit loss of cell numbers in lymphoid tissue or weight loss reflective of GVHD in this strain combination (Figure 5B,C). Notably, although few Vα2^+ CD8^+ CD45.1^+ T cells were present in non-PTC treated

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**Figure 5.** Persistence of non-antihost reactive donor T cells only in recipients treated with PTC and protected from GVHD. Recipient C3H.SW were conditioned with a single dose of 10.5 Gy TBI on day 0. 2 x 10^6 CD4^+ and CD8^+ B6-CD45.1^+ T cells with or without 2 x 10^6 B6 CD45.2^+ OT-I CD8 T cells were coinfused together with 6 x 10^6 B6-CD45.1^+ TCD-BM (n = 4 mice per group). Some groups received 66 mg/kg i.p. injection of PTC on day 3. Recipient tissues were harvested on day 28 and assessed for total cell numbers and numbers of OT-I T cells. (A) Total numbers of Vα2^+ CD8^+ CD45.1^+ (OT-I) T cells in lymph nodes of mice at day 29. P = .003 and P = .03 for T cells only/T cells + PTC, and T cells + OT-I/T cells + OT-I + PTC, respectively. (B) Total lymph node cell numbers at day 29 after transplantation. P = .001 and P = .03 for T cells only/T cells + PTC and T cells + OT-I/T cells + OT-I + PTC, respectively (1 of 3 representative experiments). (C) Mean percentage weight loss versus initial starting weight of each group of transplant recipients. T cells only versus T cells + PTC: P = .09; T cells + OT-I versus T cells + OT-I + PTC: P = .11.

**Figure 6.** Non–antihost reactive donor T cells are present >6 weeks after PTC treatment in allogeneic HSCT recipients protected from GVHD. Recipient C3H.SW were conditioned with a single dose of 10.5 Gy TBI on day 0. 2 x 10^6 CD4^+ and CD8^+ B6-CD90.1^+ T cells with or without 2 x 10^6 B6 CD45.2^+ CD90.2^+ OT-I CD8 T cells were coinfused together with 6 x 10^6 B6-CD45.1^+ TCD-BM (n = 4 mice per group). Some groups received 66 mg/kg i.p. injection of PTC on day 3. (A) Total numbers of splenic Vα2^+ Vβ15^+ CD8^+ CD45.1^+ CD90.1^+ (Ly9.1, which is expressed by C3H.SW T cells, was not detected on these cells; data not shown) OT-I T cells in spleens of mice day 44 after transplantation. (B) Mean percentage weight loss versus initial starting weight of each group of transplant recipients. T cells only versus T cells + PTC: P < .0001; T cells + OT-I versus T cells + OT-I + PTC: P < .0001. (C) Mean clinical score of each transplantation group based on monitoring changes including posture, fur texture, and alopecia as described in Methods; of 3 representative experiments. T cells only versus T cells + PTC: P < .0001; T cells + OT-I versus T cells + OT-I + PTC: P = .0005.
recipients undergoing GVHD (graph percentages represent <10 gated events per mouse), these T cells were readily detectable in lymph nodes in recipients treated on day 3 with 66 mg/kg PTC ~1 month after HSCT (Figure 5A). The ability of PTC to facilitate persistence of non–antihost OT-I T cells while concomitantly inhibiting GVHD was corroborated in an independent transplantation, in which recipients were examined >6 weeks after HSCT (Figure 6). PTC treatment again clearly prevented weight loss and clinical signs induced by GVHD (Figure 6B,C). Importantly, only in PTC-treated animals were transplanted Vß5+ CD8+ CD45.1+ CD90.1+ OT-I cells present, indicating that these cells survived the PTC treatment regimen, which ameliorated GVHD (Figure 6A).

**DISCUSSION**

Bacterial, viral, and fungal infections are recurring complications associated with the diminished immune function after HSCT. Immune deficiency after transplantation can arise as a consequence of conditioning, GVHD prophylaxis, and GVHD-associated suppression. The use of methotrexate and cyclosporine to inhibit donor antihost allo-reactive responses leading to GVHD after HSCT has been the standard of care for more than 2 decades [45,46]. Strategies that could block GVHD-inducing T cells while sparing other populations to provide immunity against pathogens and tumor antigens after transplantation would represent an advance in treatment. Recent clinical trials have provided evidence that administration of cyclophosphamide after T cell–replete allogeneic HSCT may be a strategy to minimize long-term immunosuppression [24]. In the present study, we investigated the question of whether PTC results in a global and equivalent diminution of all dividing T cells, or whether antihost alloantigen-specific donor T cells may be more effectively depleted than other populations. The findings illustrate that, although nonhost allo-antigen reactive donor T cells undergo some division early after transplantation in the lymphopenic setting, cyclophosphamide can be administered at days 3 to 4 in a manner that predominantly spares such cells while concomitantly depleting a sufficient level of antihost allo-reactive T cells to ameliorate GVHD.

In the 1970s, PTC administration was reported to promote tolerance to allogeneic MHC-mismatched skin grafts in mice [18]. These results were followed by the observation that particular Vß TCR families were deleted after MHC-matched allogeneic skin grafting and cyclophosphamide infusion was performed, consistent with the hypothesis that the alloreactive T cell response was inhibited by PTC [47]. Notably, these studies were not conducted under lymphopenic conditions. Subsequent work by Luznik and Fuchs demonstrated that PTC could promote engraftment after nonmyeloablative HSCT, supporting the notion that antihost allo-antigen reactive donor T cells elicited after transplantation could be eliminated [21]. Accordingly, the use of PTC in clinical allogeneic stem cell transplantation was performed based on the premise that this method of GVHD prophylaxis would specifically deplete activated donor antihost–specific T cells, as initially proposed by Nomoto et al. after studies involving nonconditioned HSCT recipients [48]. However, whether or not PTC has a greater influence upon reducing host allo-antigen–specific activity compared with nonhost antigen reactive T cells undergoing LIP, which is signaled by excess cytokine (eg, IL-7, IL-15) because of the marked loss of cytokine receptor binding T cells, has not been examined. Although the abrogation of GVHD is a key goal after AH SCT, the utility of PTC would be diminished if a more global deletion of cells irrespective of their capacity to induce GVHD simultaneously took place.

When CFSE-labeled B6 donor T cells were transplanted into lethally conditioned allogeneic minor histocompatibility–mismatched C3H/Sl mouse recipients, a large percentage of rapidly dividing cells were identified. After PTC (66 mg/kg total dose) administration, most of these rapidly dividing cells were eliminated and GVHD was significantly ameliorated. To assess how lymphopenic signals alone or together with antigen signals influenced susceptibility of transplanted T cells to PTC exposure, the addition of multiple TcR transgenic CD8+ T cell populations were included together with polyclonal donor CD8+ T cells. One population, OT-I, possesses a TcR specificity generated by engineering a RAG-1 marrow to express a TcR Vß and Vß segment that yields a specificity for a peptide of hen egg white lysozyme (an antigen not normally found in the mouse) presented by the MHC class I H2 Kb molecule. The second CD8 TcR transgenic population (ie, PMEL-1 with specificity for the gpl00 melanoma antigen) is also presented by MHC Kd, and is an antigen that is expressed in the skin and ocular tissue at low levels. Notably, previous work by others reported more rapid expansion kinetics under lymphopenic conditions of OT-I CD8+ T cells (~1 division per 24 hours) versus PMEL-1 CD8+ T cells (~1 division per 34 hours) [42,44], and these findings were corroborated in the present work. When cotransplanted into lethally conditioned syngeneic recipients, neither CD8+ T cell transgenic population was significantly diminished by the PTC dose employed as assessed by CFSE division and cell number (Figures 3, 4). However, the more rapid rate of LIP by OT-I than Pmeln-1 T cells did appear to consistently, although modestly, increase their susceptibility to PTC. We interpret these findings to indicate that PTC susceptibility was markedly higher for T cells dividing more rapidly (multiple times per day) (ie, as a result of allo-antigen–stimulated activation after transplantation). Consistent with such a hypothesis was the finding that when OT-I CD8 T cells were subsequently mixed with splenocytes from transgenic B6-OVA–producing mice, the more rapid rate of OT-I proliferation demonstrated by dye dilution was accompanied by a large reduction in CD8 OT-I cell number after PTC administration (Figure 4B). It is interesting that freshly obtained OT-I T cells expressing a predominantly naive (ie, CD44 CD62L+ [Figure S2]) phenotype become highly susceptible to day 3 and 4 PTC administration after antigen exposure because naive donor T cells appear to mediate severe GVHD responses, and, therefore, deletion of responsive naive T cells would presumably be important as well as effective for GVHD prevention [10,49].

A key benefit of post-transplantation usage of cyclophosphamide in hematopoietic cell transplants is that the pro-drug 4-hydroxycyclophosphamide is not converted to the alkylating phosphoromide mustard compound in cells expressing high levels of aldehyde dehydrogenase, which includes hematopoietic stem cells, thus removing the necessity for additional stem cell infusion [50-54]. Although cyclophosphamide–induced alkylation can occur throughout the cell cycle, it most effectively kills rapidly proliferating cells, especially those in the G1 and S phases. Resistance can result from several pathways, including increased drug efflux, reaction of the drug with thiols (eg, glutathione), as well as increased DNA repair time. Successful DNA repair leading to survival requires the coordination of a number of protein factors to the damaged area, access to the strands,
and sufficient time [55]. Because, to some extent, both unactivated and activated T cells convert cyclophosphamide to the alkylating form, it is reasonable to propose that the more rapid the expansion kinetics by a T cell population (eg, one driven by allogeneic antigen to divide multiple times per day) compared with the division stimulated by cytokines under lymphopenic conditions (ie, ~ once per 18 to 36 hours), the less time there will be for the former to repair damage caused in G1/S phase, resulting in death due to failure to replicate damaged DNA [56,57]. Indeed, a consistent observation in our studies was the capacity of our PTC treatment regimens to prevent induction of GVHD mediated by donor T cells reactive with host allo-antigen epitopes (Figures 1,5,6,S1). The ability to enhance DNA repair pathways in selected cell populations could, therefore, provide approaches to minimize and/or abrogate the loss of slowly dividing cells with the objective to augment immediate immune responsiveness and protection in recipients after allogeneic hematopoietic stem cell transplantation.

The present study has important implications with regard to immune function and/or reconstitution after T cell replete allogeneic HSCT. Survival and responsiveness by those infused T cells, which are not host allogeneic transplantation antigen reactive, is crucial to provide patients with immediate immune function and protection against both pathogens and residual hematopoietic tumor cells. The findings here support the notion that the antigen and cytokine signals present, which can rapidly induce T cell proliferation post-HSCT, will have a major impact on determining the T cell populations that persist after administration of day 3 and/or 4 post-transplantation cyclophosphamide, and accordingly, the cells that can be exploited via vaccination or other means to provide rapid adaptive immune function to protect individuals from opportunistic infections, as well as relapse after transplantation. A critical issue to address concerns the fate of anti-tumor reactive T cells in recipients treated with PTC. We anticipate their survival will be influenced by the signals noted above including the T cell receptor affinity of the clones involved which will influence the kinetics of division and thereby the time available for DNA repair post-allylation. We posit that together with the drug concentration administered, non-Bcl-2 dependent and application of strategies that diminish nonhost allo-antigen—specific T cell proliferation and promote DNA repair can better protect “desired” T cells and, thereby, successfully facilitate more rapid and effective immune function in such patients.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2013.06.019.

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