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Original Article

Sigle Agent of Posttransplant Cyclophosphamide Without Calcineurin Inhibitor Controls Severity of Experimental Chronic GVHD

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Chronic graft-versus-host disease (GVHD) is a major cause of late death and morbidity following allogeneic hematopoietic cell transplantation (HCT), but its pathogenesis remains unclear. Recently, haplo-identical HCT with post-transplant cyclophosphamide (Haplo-HCT with PTCY) was found to achieve a low incidence rate of acute GVHD and chronic GVHD. However, while the pathogenesis of acute GVHD following Haplo-HCT with PTCY has been well investigated, that of chronic GVHD remains to be elucidated, especially in HLA-matched HCT with PTCY. Based on its safety profile, PTCY is currently applied for the human leucocyte antigen (HLA)-matched HCT setting. Here, we investigated the mechanisms of chronic GVHD following HLA-matched HCT with PTCY using a well-defined mouse chronic GVHD model. PTCY attenuated clinical and pathological chronic GVHD by suppressing effector T-cells and preserving regulatory T-cells compared with a control group. Additionally, we demonstrated that cyclosporine A (CsA) did not show any additional positive effects on attenuation of GVHD in PTCY-treated recipients. These results suggest that monotherapy with PTCY without CsA could be a promising strategy for the prevention of chronic GVHD following HLA-matched HCT.

Key words: GVHD, posttransplant cyclophosphamide, hematopoietic cell transplantation, HLA-identical

Control of graft-versus-host disease (GVHD) is critical for the success of allogeneic stem-cell transplantation. For nearly 40 years, the combination of methotrexate (MTX) and a calcineurin inhibitor (CI) was the main approach for GVHD prevention [1]. Even with such prophylaxis, clinically significant acute GVHD, chronic GVHD or both develop in more than half of recipients following allogeneic hematopoietic cell transplantation (allo-HCT) [2,3]. These conditions can cause significant complications and even death after allo-HCT. Recent advances in allo-HCT have enabled human leucocyte antigen (HLA) haplo-identical HCT using post-transplant cyclophosphamide (PTCY) and CI in the clinical setting [4-6]. The rationale for Haplo-HCT with CI+PTCY is that high-dose cyclophosphamide has been shown to spare donor hematopoietic stem cells and regulatory T cells (Tregs) after allo-HCT [7-10]. In fact, CI+PTCY showed a low incidence of GVHD with fewer alloreactive effector T cells, greater Treg cell expansion and more rapid B cell recovery [9,11-13]. Moreover, a study comparing three strategies for HLA-matched reduced-intensity conditioning HCT — *i.e.*, CI+MTX+bortezomib, CI+MTX+maraviroc and CI+PTCY — in a phase 2 clinical trial initiated by the Blood and Marrow Transplant Clinical Trials

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Network (BMT CTN) found that CI+PTCY was the most promising regimen [14]. A subsequent randomized phase 3 clinical trial comparing CI+MTX and CI+PTCY for patients undergoing HLA-matched HCT with reduced-intensity conditioning found that the CI+PTCY group showed significantly longer GVHD-free, relapse-free survival compared to the CI+MTX group [15]. Given these results, CI+PTCY is now applied in the HLA-matched HCT setting.

Studies using a mouse model of haplo-HCT with CI+PTCY also reproduced phenomenona observed in human HCT — namely, that activated alloreactive T cells are selectively depleted and Tregs are preserved by CI+PTCY, because Tregs expressed relatively high levels of aldehyde dehydrogenase (ALDH) compared with conventional CD4 T cells (Tcons) and received fewer damages from PTCY [16, 17]. These results showed that the mechanism underlying the efficacy of CI+PTCY against GVHD is based on not only depletion of donor alloreactive T cells but also preservation of donor Tregs. In addition to acute GVHD prophylaxis, the low incidence of chronic GVHD is noteworthy [18-21]. In recipients of peripheral blood stem cell grafts, the incidence of moderate to severe chronic GVHD is significantly lower in Haplo-HCT with CI+PTCY recipients even compared with fully HLA-matched unrelated donors recipients [11, 22, 23]. Tregs play an important role in the pathogenesis of chronic GVHD, and impaired reconstitution of Tregs can result in loss of tolerance to allo-immune reactions, leading to the development of chronic GVHD [24, 25]. Previously, we reported that Tregs arisen from donor BM-derived progenitor cells maintained the long-term peripheral Treg pools and cyclosporine A (CsA) compromised Treg homeostasis in the periphery and thymus [26]. Still, the effects of CI+PTCY on the GVHD pathogenesis in an HLA-matched setting are not well documented and need to be elucidated.

In the current study, we used a well-defined MHC matched/minor antigen (miHA) incompatible mouse model of chronic GVHD that mimics human HLA-matched HCT, and investigated the role of CI+PTCY in the pathophysiology of chronic GVHD. We also evaluated the effects of CI+PTCY on immune cells and Tregs in the context of chronic GVHD.

Methods

Bone marrow transplantation. Female recipient BALB/c (H-2^d) mice were purchased from CLEA Japan (Tokyo). Female donor B10.D2 (H-2^d) mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained under specific pathogen-free condition and used at 8-12 weeks of age. All animal experiments were performed according to the regulations of the Institutional Animal Care and Research Advisory Committee of the Okayama University Advanced Science Research Center. Balb/c mice received a single dose of 5.8 Gy X-ray total body irradiation, and on the same day, were injected with 2×10^6 spleen T cells or 8×10^6 splenocytes and 8×10^6 T cell-depleted bone marrow cells (TCD-BM) from syngeneic Balb/c or allogeneic B10.D2 mice through the tail vein [27]. The T-cell depletion and purification were performed with CD90.2 Microbeads or a Pan T Cell Isolation Kit using an Auto MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The Treg isolations were performed with a $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec) using an Auto MACS system. Cyclophosphamide (CY) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and cyclosporine A (CsA) was purchased from AdipoGen (San Diego, CA, USA). CY was dissolved in saline at a concentration of 3.33 mg/ml and administered to the recipients by peritoneal injection at a dose of 33 mg/kg at days 3 and 4 post-HCT. CsA was given as a suspension in carboxymethylcellulose sodium salt (CMC) (Sigma-Aldrich) at a final concentration of 0.2% CMC, and administered to recipients by peritoneal injections at 20 mg/kg daily from day 0 to day 28 post-HCT.

Evaluation of chronic GVHD. After HCT, recipient mice were weighed and scored for systemic or skin manifestations of GVHD. The skin GVHD scores were determined as described previously [28]. Briefly, the following scoring system for fur texture was determined as ruffing, areas, skin lesions of alopecia were utilized. The minimum score was 0, and the maximum was 4.0.

Tissue histopathology. Shaved skin from the interscapular region (1-2 cm^2), salivary glands and liver specimens from recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin. Masson trichrome staining was used for to evaluate fibrosis in skin

specimens. Skin slides were scored on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes and follicular dropout (0-2 for each category; the maximum score was 10) [29]. Slides of salivary gland were also scored on the basis of atrophy and inflammation (0-3 for each category; the maximum score was 6) [26]. All slides were scored by a pathologist (T.T.) in a blinded manner.

Preparation of lymphocytes single cell suspension and flow cytometry. Systemic lymph nodes (axillary and inguinal) and spleens were mechanically disrupted. After lysis of red blood cells, single cell suspensions were resuspended in FACS wash buffer (2% bovine serum albumin in PBS). The cells were then stained with the following conjugated monoclonal antibodies (mAbs): FITC-conjugated anti-CD8a (53-6.7), anti-Qa2 (69H1-9-9), PE-conjugated anti-CD25 (PC61.5), APC-conjugated anti-Foxp3 (FJK-16s), eFluor®450-conjugated anti-CD4 (GK1.5), PE-Cyanine7-conjugated anti-CD24 (M1/69), APC-conjugated anti–IL-17A (eBio17B7), PE-Cyanine7-conjugated anti–IFN-γ (XMG1.2) and control Abs from eBioscience (Affymetrix Japan K.K., Tokyo). For intracellular staining, a Foxp3/Transcription Factor Staining Buffer Set purchased from eBioscience was used according to the manufacturer's instructions. Cells were stimulated in RPMI-1640 containing 10% fetal bovine serum (FBS) with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) at 37°C for 3 h. Cells were then incubated with Golgi Stop (BD Bioscience) for additional 2 h incubation, and stained with Abs. Total cells were adjusted to 1×10^6/ml in culture Cells were also analyzed with a MACSQuant flow cytometer using FlowJo software.

Statistical analyses. Group comparisons of skin chronic GVHD scores, pathology scores, and cell populations were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Survival was evaluated using the log-rank test. All data were analyzed using GraphPad Prism software (version 5.0). Values of $p < 0.05$ were taken to indicate statistical significance.

Results

PTCY ameliorated chronic GVHD irrespective of cyclosporin A. First, to evaluate the effects of PTCY on murine chronic GVHD, we used a common MHC-matched, miHA-incompatible murine BMT model (B10.D2 into BALB/c) that represents human chronic GVHD. Sublethally irradiated BALB/c mice were transplanted with 2×10^6 spleen T cells and 8×10^6 TCD-BM cells from syngeneic Balb/c or allogeneic B10.D2 mice. Allogeneic recipients showed severe weight loss, higher clinical chronic GVHD scores and lower survival rates than the syngeneic recipients (Fig. 1A-C). We then evaluated the efficacy of PTCY with or without CsA for the prevention of murine chronic GVHD; the results are shown in Fig. 2A along with those for human Haplo-HCT with CI+PTCY. The recipient mice were divided into 5 groups as follows: syngeneic and allogeneic recipients treated with vehicle control (vehicle group), CsA alone (CyA group), PTCY alone (PTCY group), and both PTCY and CsA (PTCY-CsA group). Recipient mice were transplanted from syngeneic or allogeneic donors as mentioned above. Then recipients were observed until day 50 after BMT and the histopathological damages were assessed. The CsA group exhibited a lower skin GVHD score compared to the vehicle group (2.5 ± 0.339 for the vehicle group vs 2.00 ± 0.726 for the CsA group: $p < 0.01$), while the PTCY group showed a significant improvement in the skin GVHD score irrespective of CsA administration, similar to the syngeneic group (2.50 ± 0.339 for the vehicle group vs 0.50 ± 0.00 for the PTCY group: $p < 0.01$; $2.50 \pm$ error for the vehicle group vs 0.50 ± 0.00 for the PTCY-CsA group: $p < 0.01$). The PTCY group and PTCY-CsA group mice developed less alopecia than the vehicle or the CsA groups, and their levels of alopecia were almost identical to those in the syngeneic group, as shown in Fig. 2C. Histological examination of skin and salivary glands, which were the main target organs of chronic GVHD, were collected from the recipient mice at day 50 after BMT. In the control vehicle group, the skin showed pathologically exacerbated GVHD manifestations with decreased fat tissues, massive invasion of donor lymphoid cells and loss of hair follicles (Fig. 2D). These changes were significantly attenuated by CY administration. The skin pathological scores in the PTCY group were significantly lower than those in the vehicle group and CsA groups (5.75 ± 0.75 for the vehicle group vs 2.00 ± 0.316 for the PTCY group: $p < 0.001$; 5.00 ± 1.00 for the CsA group vs 4.40 ± 0.245 for the PTCY-CsA group: $p < 0.01$; Fig. 2E). The skin pathological score in the PTCY-CsA group was also significantly lower than those in the vehicle and CsA

groups, but higher than that in the PTCy-vehicle group (2.00 ± 0.316 for the PTCY group vs 4.40 ± 0.245 for the PTCY-CsA group: $p < 0.05$). The pathological score of the salivary glands in the PTCY-CsA group tended to be higher than that of the PTCY group, but there were no significant differences. These data indicate that PTCY ameliorates both clinical and histological chronic GVHD, and CsA confers no additive benefit over PTCY monotherapy.

PTCY administration attenuated chronic GVHD by suppressing the IFN-γ$^+$ and IFN-γ$^+$ IL-17A$^+$ T cell expansion. We next evaluated the efficacy of PTCY and CsA administration on effector T cell functions in the murine chronic GVHD model. Previously, we reported that IFN-γ$^{-/-}$ or IL-17A$^{-/-}$ donor T cells showed significantly reduced GVHD damage compared to the control mice and that CD4 T cells positive for IFN-γ, IL-17A, or both play important roles on the chronic GVHD severity. In our present analysis, we also determined the cytokine production of donor T cells from peripheral lymph nodes (pLNs) in each group (Fig. 3A). On day 28 after BMT, IFN-γ$^+$ IL-17A$^-$ and IFN-γ$^+$ IL-17A$^+$ CD4 T cells from pLNs of the vehicle group were increased and those cells were less frequent in the PTCY or PTCY-CsA group, but there were no significant differences between the PTCY and PTCY-CsA group. There were no apparent differences in the numbers of IFN-γ$^-$ IL-17A$^+$ CD4 T cells among groups (Fig. 3B). We also assessed the IFN-γ and IL-17A production on CD8 T cells, but found no significant change in the allogeneic groups (data not shown). These results suggest that PTCY reduces chronic GVHD damage by depleting cytokine-producing effector IFN-γ$^+$ IL-17A$^-$ and IFN-γ$^+$ IL-17A$^+$ CD4 T cells. CsA did not show any additive effects on the depletion of alloreactive T cells by PTCY.

Cyclosporine A did not affect the PTCY-induced regulatory T cell expansion in a murine chronic GVHD model. To elucidate the immunosuppressive mechanisms of PTCY, we focused on Tregs. PTCY selectively depletes activated alloreactive T cells and preserves Tregs, based on the findings that Tregs were less

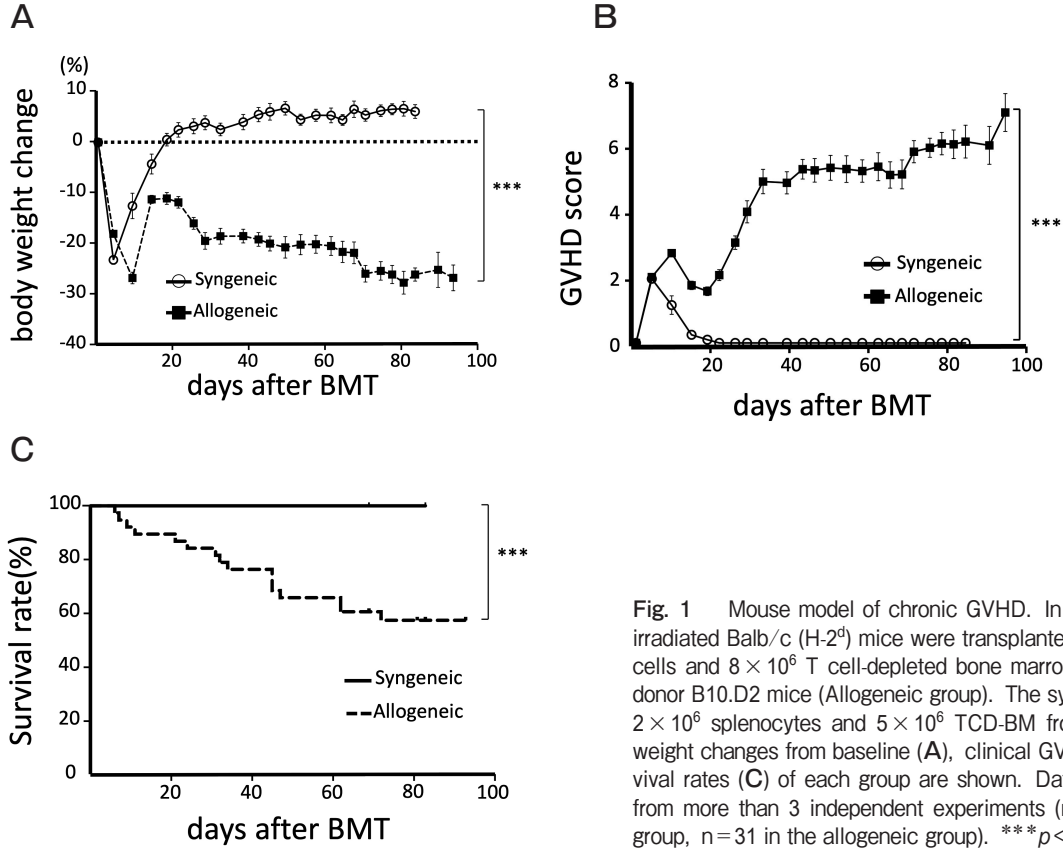

Fig. 1 Mouse model of chronic GVHD. In this model, sublethally irradiated Balb/c (H-2^d) mice were transplanted with 2×10^6 spleen T cells and 8×10^6 T cell-depleted bone marrow cells (TCD-BM) from donor B10.D2 mice (Allogeneic group). The syngeneic group received 2×10^6 splenocytes and 5×10^6 TCD-BM from Balb/c mice. Body weight changes from baseline (**A**), clinical GVHD scores (**B**) and survival rates (**C**) of each group are shown. Data shown are combined from more than 3 independent experiments (n = 12 in the syngeneic group, n = 31 in the allogeneic group). ***$p < 0.005$.

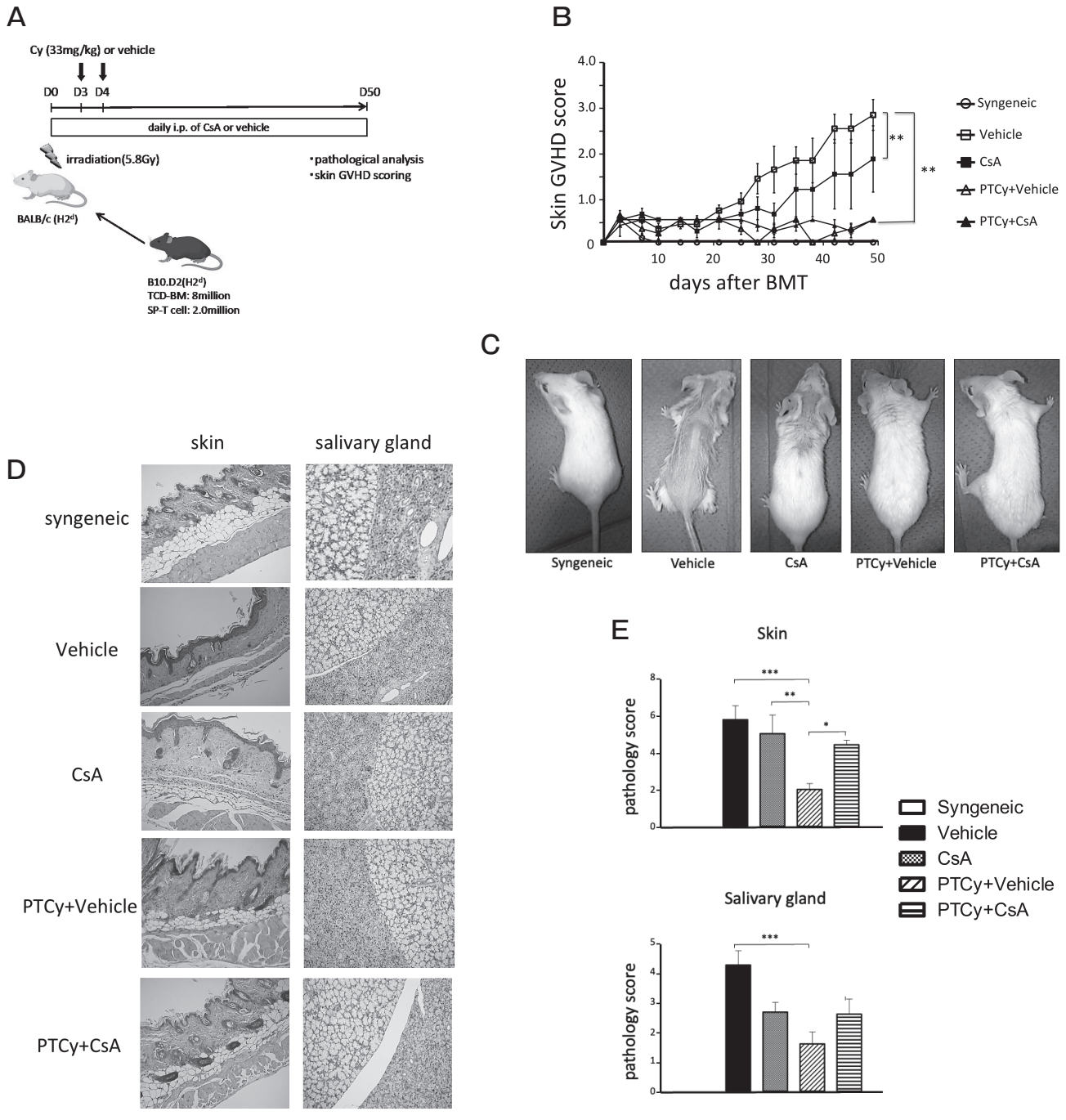

Fig. 2 PTCY dramatically improved clinical and pathological chronic GVHD scores irrespective of CsA administration. Sublethally irradiated recipient Balb/c mice were transplanted with 2×10^6 spleen T cells and 8×10^6 TCD-BM from donor B10.D2 mice. CsA was administered to the recipients by peritoneal injection at a dose of 20 mg/kg daily from day 0 to day 50. For recipients in the PTCY-treatment group, CPA was administered by peritoneal injection at a dose of 33 mg/kg on days 3 and 4 post-HCT. The skin chronic GVHD score was recorded over time and pathological analysis was performed on day 50 on BMT for each group. (**A**) Schema of the BMT model with PTCY is shown. (**B**) Skin GVHD scores after BMT are shown. (**C**) Representative images of the appearance of mice in each group at 50 days after BMT. (**D**) Representative pathological images of H&E stained slides are shown. (**E**) Pathological scores of skin specimens and salivary glands on day 50 are shown. The means (± SE) of each group are shown. Data shown are from 1 representative of 2 independent experiments (n = 3–6 in each group). NS: not significant. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$.

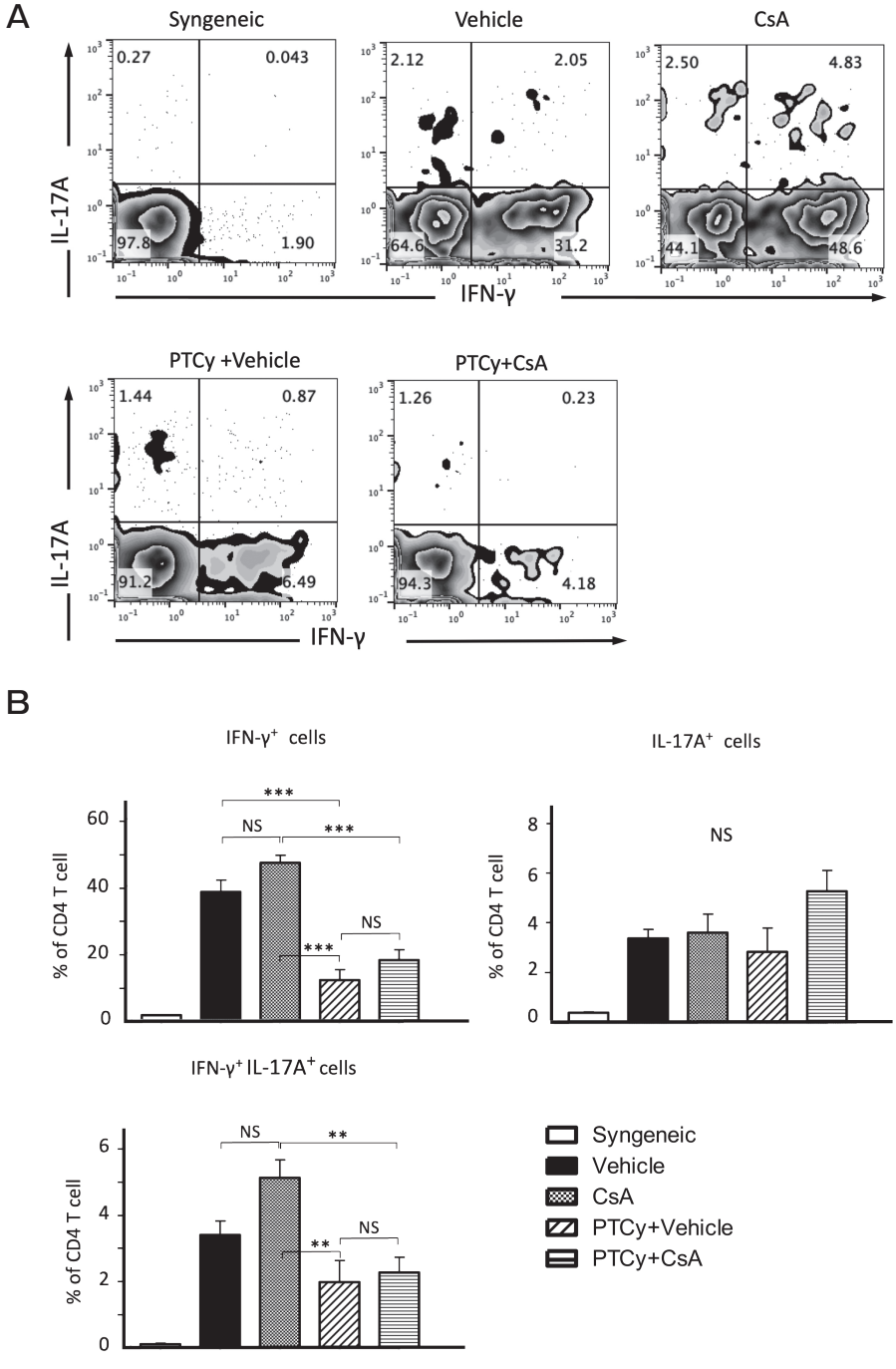

Fig. 3 PTCY suppressed the expansion of Th1, Th17 and Th1/Th17 cells in chronic GVHD mice. (**A**–**B**) Sublethally irradiated recipient Balb/c mice were transplanted from donor B10.D2 mice. PLNs were collected on day 28 after BMT from recipient mice and processed into single-cell suspensions. Cells were stimulated with PMA and ionomycin at 37°C for 3 h followed by addition with Golgi Stop and incubation for an additional 2 h. Anti-IL-17A and anti-IFN-γ mAbs were used to assess the cell populations. (**A**) Representative FACS plots of Th1 (IFN-γ^+IL-17A$^-$), Th17 (IFN-γ^- IL-17A$^+$) and Th1/Th17 (IFN-γ^+ IL-17A$^+$) cells among CD4$^+$ T cells. (**B**) The percentages of Th1, Th17 and Th1/Th17 cells among CD4$^+$ T cells are shown. The means (± SE) of each group are shown. Data shown are combined from 2 independent experiments (n = 3–6 in each group). NS: not significant. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$.

affected by PTCY due to their relatively high expression of aldehyde ALDH, which reduces damages from PTCY, resulting in Tcon eliminations. We measured the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in the pLNs using the same chronic GVHD model as used in the above experiments. On day 14 after BMT, when clinical GVHD symptoms were not yet apparent, we could find no clear differences in the proportions of Tregs in CD4 T cells from each group (Fig. 4A, B). On day 28 after BMT, when clinical GVHD had developed, Treg proportions in the pLNs were reduced in the vehicle and CsA groups, although Treg proportions in the PTCY group and the PTCY-CsA group were significantly higher than those in the other three groups (7.76 ± 1.76 for the vehicle group vs 21.0 ± 1.58 for the PTCY group: $p < 0.001$; 4.06 ± 1.07 for the CsA group vs 29.33 ± 2.57 for the PTCY-CsA group: $p < 0.001$) (Fig. 4A, 4B). There were no significant differences of Treg proportions between the vehicle group and the CsA group or between the PTCY group and the PTCY-CsA group. These data suggest that CsA does not affect the PTCY-mediated Treg expansion during the chronic GVHD development.

Depletion of $CD4^+$ $CD25^+$ T cells from donor spleen had no effect on the Treg expansion after HCT. In this study we determined the effects of PTCY on both elimination of effector T cells and preservation of Tregs. We showed that PTCY preserved the expansion of the Tregs on day 28 after HCT. We next asked whether the increased Tregs were derived from the donor bone marrow progenitor cells or from the existing, transplanted Tregs of the spleen. To evaluate the origin of the expanded Tregs, we depleted the $CD4^+$ $CD25^+$ T cells from the donor splenocytes and transplanted them with TCD-BM to the recipient mice. Harvested cells from the spleen and pLNs on day 28 post-BMT were analyzed for Tregs and cytokine production of effector T cells. The skin GVHD scores of the PTCY group were significantly lower than that of the vehicle group (2.123 ± 0.214 for the vehicle group vs 0.50 ± 0.177 for the PTCY group: $p < 0.01$) (Fig. 5A). The frequencies of the Tregs in the PTCY group were significantly higher than those in the syngeneic and vehicle groups (10.1 ± 0.265 for the syngeneic group vs 21.6 ± 1.04 for the PTCY group: $p < 0.01$; 6.21 ± 2.33 for the vehicle group vs 21.6 ± 1.04 for the PTCY group: $p < 0.001$) (Fig. 5B). IFN-γ^+ IL-$17A^-$ cells were increased in pLNs from the vehicle group, and were detected less frequently in pLNs from the PTCY group. We also evaluated the proportion of IFN-γ^+ IL-$17A^+$ cells among CD4 T cells, and found that the CsA group had a higher proportion of IFN-γ^+ IL-$17A^+$ cells than the PTCY-vehicle group. These results — the expansion of Tregs and reduction of IFN-γ^+ IL-$17A^-$ CD4 T cells in pLNs — were similar to the findings in the Treg-maintained BMT setting, as shown in Fig. 3 and 4. Collectively, these results suggest that Tregs expansion in the periphery of recipients after PTCy could be derived from the donor stem cells. Furthermore, the immunosuppressive effects of PTCY in an MHC-matched, miHA-incompatible murine BMT model representing human chronic GVHD were shown to be dependent on both the depletion of effector T cells and expansion of Tregs.

Discussion

In this study, we demonstrated that PTCY was effective in HLA-matched transplantation and attenuated both clinical and pathological chronic GVHD using a well-defined, chronic GVHD mouse model that resembles human GVHD. PTCY suppressed IFN-γ single-positive (IL-17^-) and IFN-γ/IL-17 double-positive cells compared with control-treated allogeneic recipients, and Tregs were increased significantly in PTCY-treated recipients. In addition, we demonstrated that CsA did not improve the reconstitution of Tregs or attenuation of GVHD in PTCY-treated recipients.

Together, a number of previous reports suggest a mechanism by which PTCY may ameliorate allogeneic immune reactions [30]. First, PTCY depletes the alloreactive proliferative effector T cells that cause organ damages [10]. Second, high-dose cyclophosphamide spares donor hematopoietic stem cells and regulatory T cells (Tregs) after HCT [9]. Third, Tregs preservation leads to healthy immune cell recovery, such as rapid B-cell homeostatic proliferation, which in turn reduces chronic GVHD incidence [13, 17]. After haplo-HCT, alloreactive T cells proliferated vigorously and depleted with PTCY. PTCY was initially developed in the setting of haplo-HSCT, and PTCY for haplo-HCT was established for both acute and chronic GVHD prevention [15, 31]. As a next step, it is necessary to determine whether PTCY is effective in HLA matched transplantation. Our data demonstrated that PTCY in an MHC-matched, mouse chronic GVHD model prevented effector T-cell expansion and protected Tregs from

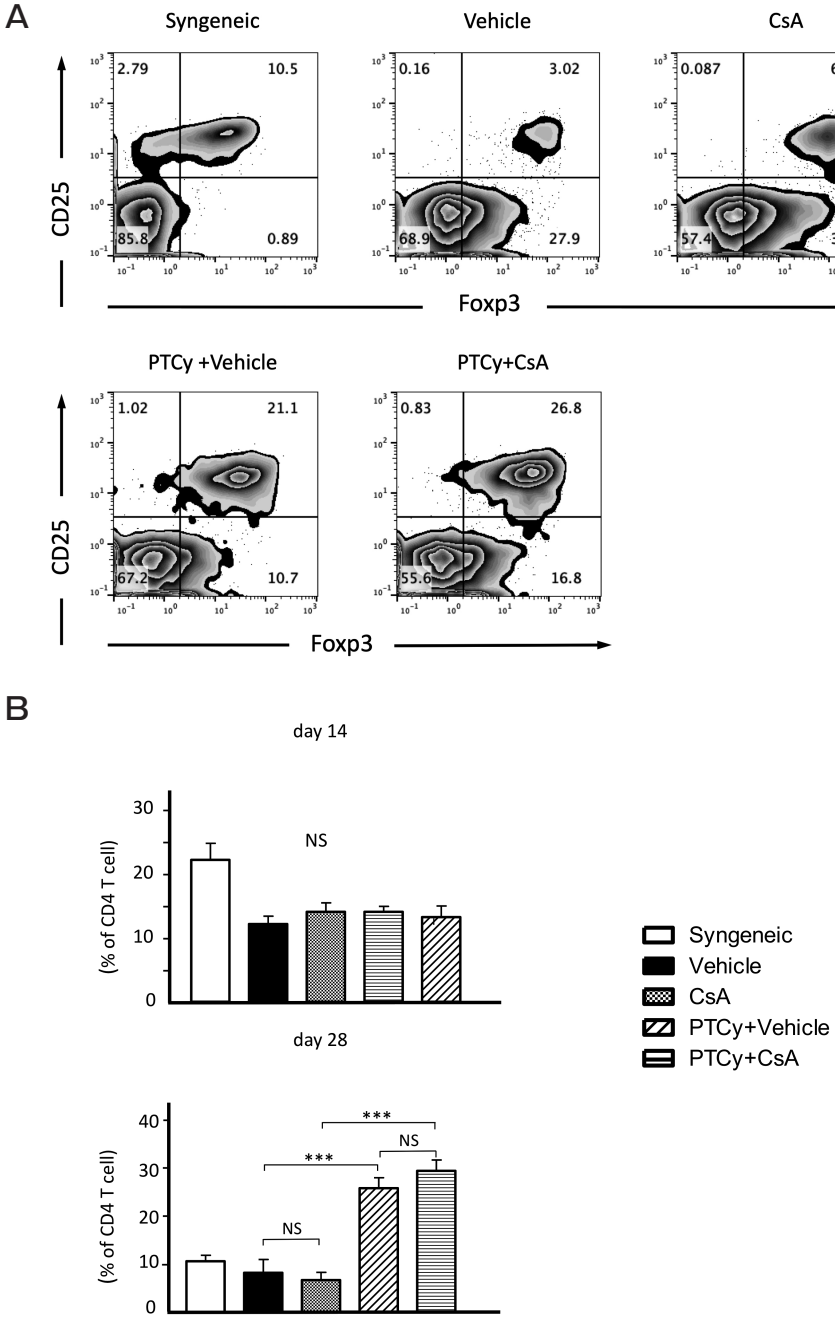

Fig. 4 PTCY administration enhanced the recovery of regulatory T cells independently of CsA treatment. (**A**–**B**) Sublethally irradiated recipient Balb/c mice were transplanted from donor B10.D2 mice. PLNs were collected at day 14 or day 28 after BMT from recipient mice and processed into single-cell suspensions. The percentages of regulatory T cells (Tregs) that were positive for anti-CD25 and anti-Foxp3 antibody among total CD4 T cells were calculated. (**A**) Representative FACS plots of $CD25^+$ $Foxp3^+$ Tregs among $CD4^+$ T cells in each group. (**B**) The percentages of Tregs among $CD4^+$ T cells are shown. The means (± SE) of each group are shown. Data shown are from 1 representative of 2 independent experiments (n = 3–6 in each group). NS: not significant. $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.005$.

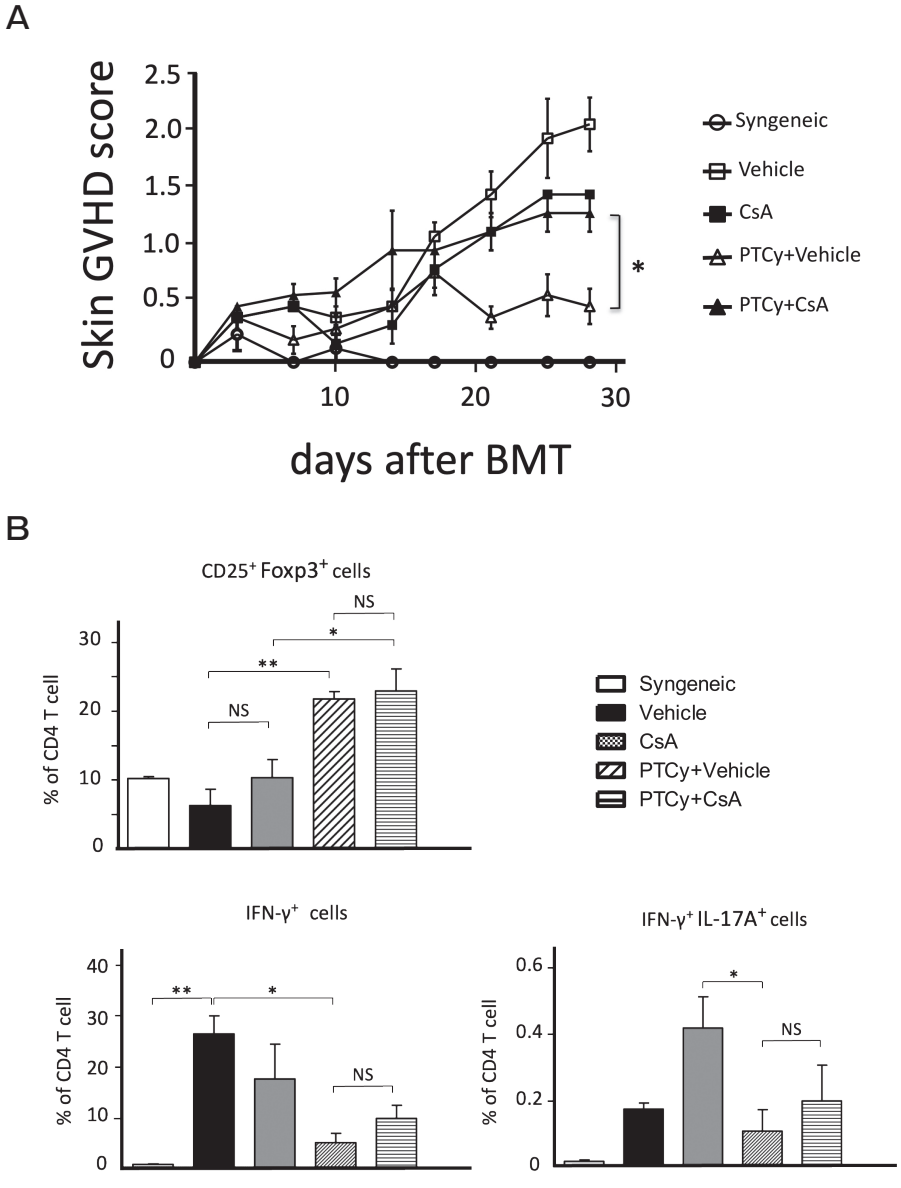

Fig. 5 Spleen derived $CD4^+$ $CD25^+$ Tregs did not affect the significant recovery of Tregs after BMT in PTCY-treated mice. (**A–B**) Sublethally irradiated recipient Balb/c mice were transplanted with 8×10^6 TCD-BM and $CD4^+$ $CD25^+$ Treg-depleted 8×10^6 splenocytes from donor B10.D2 mice. (**A**) Chronological changes of skin GVHD scores of recipient mice. (**B**) PLNs were collected at day 28 after BMT from recipient mice and processed into single-cell suspensions. Percentages of regulatory T cells (Tregs), IFN-γ^+ IL-$17A^-$ cells and IFN-γ^+ IL-$17A^+$ cells among the CD4 T cells were calculated. The means (± SE) of each group are shown. Data shown are from 1 representative of 2 independent experiments (n = 3–6 in each group). NS: not significant. *$p < 0.05$ and **$p < 0.01$

GVHD damages. Ganguly *et al.* showed that the prophylactic efficacy of PTCY against acute GVHD is dependent on donor Tregs, and BMT with grafts depleted of Tregs showed loss of efficacy of PTCY [10]. In this study, Tregs were increased significantly in PTCY-treated recipients during the late phase after HCT and depletion of $CD4^+$ $CD25^+$ T cells from donor spleen had no efficacy on the Treg expansion after HCT. These findings suggest that Tregs expansion in the periphery of recipients after PTCY could be derived from the donor stem cells, as we previously showed in the setting of haplo-HSCT [17]. Taken together, these data

revealed that PTCY functioned even in the MHC-matched setting, and attenuated chronic GVHD via suppression of Th17/Th1 and enhancement of Treg reconstitution.

CI administration is a historically well-established immunosuppressant in allo-HCT [4-6,14,15]. Although CI is a standard method usually used to prevent acute GVHD and to treat acute/chronic GVHD, CI could not prevent chronic GVHD, for which the occurrence rate was around 30% to 60% [3]. Addition of CsA is clinically used to prevent GVHD with PTCY in Haplo-HCT, but its effects have not been well elucidated in the setting of HLA-matched HCT with PTCY. Previously we assessed whether CsA affects the peripheral Treg pool after allogeneic HCT and revealed that long-term use of CsA impairs reconstitution of BM-derived Tregs and increases the liability to chronic GVHD [26]. Moreover, in the haplo-mismatch setting, early administration of CI stopped allo-reactive T-cell exhaustion that expressed TOX, a master regulator, to promote differentiation of transitory exhausted T cells, leading to preserved allo-immune responses and inhibited tolerance induction [32]. In this study, we revealed that there were no significant differences of Treg proportions between the PTCY group and the PTCY-CsA group. Thus CsA does not affect the PTCY-mediated Treg expansion after HLA-matched HCT.

This study had several limitations. First, we did not address other types of GVHD, including various chronic GVHD models [33]. Also, the details of the cytokine phenotypes, alloreactive T-cell exhaustion patterns, Treg phenotypes, B-cell recovery and other immune cell-related chronic GVHD in target tissues were not determined [17]. Finally, recent advances in our understanding of GVHD suggest that tissue environments such as tissue homeostasis or stem cell regeneration could contribute to GVHD severity [34]. However, we did not investigate these factors in this study.

A previous study reported that leukemia patients who received HLA-matched HCT under remission using a reduced intensity of conditioning regimen and PTCY with CI and MMF showed superior overall survival with decreased treatment-related mobidility, including acute and chronic GVHD, and similar levels of leukemia relapse to patients receiving CI and sMTX as a GVHD prophylaxis [15]. Still, disease statuses such as non-complete remission are a major obstacle for patients receiving HCT, and it needs to be addressed whether tolerance of allogeneic immune reaction by PTCY can cure leukemia relapse and infectious events [12,35]. These points will need to be carefully elucidated in future studies. In conclusion, our study yielded basic but important findings on the effects of PTCY in a chronic GVHD animal model. Namely, PTCY was observed to preserve immuno-suppressive Tregs and suppress allogeneic effector T-cell expansion. These results should inspire further pathophysiological insights into the effects of PTCY on GVHD.

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