Bone Marrow Transplantation (2020) 55:787–795
https://doi.org/10.1038/s41409-019-0733-8

ARTICLE

Short-term KRP203 and posttransplant cyclophosphamide for graft-versus-host disease prophylaxis

Emi Yokoyama[1] · Daigo Hashimoto[1] · Eiko Hayase[1] · Takahide Ara[1] · Reiki Ogasawara[1] · Shuichiro Takahashi[1] · Hiroyuki Ohigashi[1] · Takahiro Tateno[1] · Yuta Hasegawa[1] · Xuanzhong Chen[1] · Takanori Teshima[1]

Received: 20 July 2019 / Revised: 15 October 2019 / Accepted: 17 October 2019 / Published online: 4 November 2019

Abstract

Posttransplant high-dose cyclophosphamide (PTCY) has been increasingly used as graft-versus-host disease (GVHD) prophylaxis after HLA-haploidentical or matched hematopoietic stem cell transplantation (SCT). However, PTCY alone is insufficient and requires additional immunosuppressants such as calcineurin inhibitors. In the current study, we evaluated effects of a novel GVHD prophylaxis with PTCY in combination with short-term KRP203, a selective agonist of sphingosine-1-phosphate receptor 1 that regulates egress of lymphocytes from the secondary lymphoid organs (SLOs) in mice. Short-term oral administration of KRP203 alone induced apoptosis of donor T cells in the SLOs and ameliorated GVHD. Administration of KRP203 significantly preserved graft-versus-leukemia effects compared to cyclosporin. A combination of KRP203 on days 0 to +4 and PTCY on day +3 synergistically suppressed donor T-cell migration into the intestine and skin, and ameliorated GVHD more potently than PTCY alone. A combination of short-term KRP203 and PTCY is a promising novel calcineurin-free GVHD prophylaxis in HLA-haploidentical SCT.

Introduction

Posttransplant high-dose cyclophosphamide (PTCY) has been increasingly used as GVHD prophylaxis in HLA-haploidentical hematopoietic stem cell transplantation (SCT) [1–6] and HLA-matched SCT [7]. Rationale of PTCY has been developed in mouse models of skin allograft several decades ago; PTCY selectively eliminates alloreactive T cells activated early after SCT, while preserving bystander T cells [8–10]. Recent studies further suggest that T-cell dysfunction and active suppression mediated by regulatory T cells (Tregs) play a critical role in GVHD prophylaxis with PTCY [11–13]. However, PTCY alone is not sufficient for GVHD prophylaxis, requiring administration of additional immunosuppressants such as calcineurin inhibitors (CNIs) and mycophenolate mofetil (MMF) following PTCY [1, 2, 14]. Chronic administration of CNIs is a risk for infections and chronic kidney disease, and disturbs reconstitution and survival of Tregs by inhibiting IL-2 signaling, that may blunt anti-GVHD effects of PTCY [13, 15–17]. Development of CNI-free GVHD prophylaxis is thus warranted.

Sphingosine-1-phosphate (S1P) is a metabolite of sphingolipid, a component of biomembrane. S1P interacts with five related G-protein-coupled receptors termed S1P receptor types 1 to 5 ($S1PR_{1\text{-}5}$). S1P modulates cellular proliferation, survival, and migration [18]. FTY720 (fingolimod) is a multi-S1PR inhibitor of $S1PR_1$ and $S1PR_{3–5}$ [19, 20]. FTY720 sequestrates T cells within the secondary lymphoid organs [21, 22]. Preclinical studies showed that administration of FTY720 ameliorated GVHD by inhibiting donor T-cell infiltration to GVHD target organs and facilitated rapid contraction of donor T-cell pool in association with activation-induced cell death [23, 24].

However, FTY720 exerts unique adverse effects, including bradycardia, hypo/hypertension, respiratory symptoms, macular edema, and renal impairment per its affinity to $S1PR_3$ [25]. These adverse effects might be

Supplementary information The online version of this article (https://doi.org/10.1038/s41409-019-0733-8) contains supplementary material, which is available to authorized users.

✉ Daigo Hashimoto
D5hash@pop.med.hokudai.ac.jp

✉ Takanori Teshima
teshima@med.hokudai.ac.jp

[1] Department of Hematology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan

accelerated in inflammatory condition during GVHD. Although endothelial cells and cardiomyocytes express both of $S1PR_1$ and $S1PR_3$, T lymphocytes express only $S1PR_1$ [26]. KRP203 acts specifically on $S1PR_1$ with a potentially milder toxicity profile and thus may be a promising agent to serve as a substitute of CNIs that is used in combination with PTCY [27, 28]. In the current study, we studied a role of a novel GVHD prophylaxis with PTCY in combination with short-term KRP203 using murine models of GVHD.

Materials and methods

Mice

Female B6 (H-2^b, CD45.2), Ly5a-B6 (H-2^b, CD45.1), and B6D2F1 (H-$2^{b/d}$, CD45.2) mice were purchased from CLEA Japan (Tokyo, Japan). Mice were 8–12 weeks of age at transplant and maintained in specific pathogen-free environment. Recipient mice were allocated randomly for each experimental group, ensuring the mean body weight in each group was similar. All animal experiments were performed in a nonblinded fashion and under the auspices of the Institutional Animal Care and Research Advisory Committee.

SCT

B6D2F1 recipients were lethally irradiated with 13.5 Gy total body irradiation, split into two doses with 4-h interval, followed by i.v. injection with 5×10^6 BM cells and 10×10^6 splenocytes from MHC-haploidentical B6 or syngeneic B6D2F1 donors on day 0.

Reagents

CY (Shionogi, Osaka, Japan) was dissolved in PBS at a concentration of 5 mg/ml and intraperitoneally (i.p.) injected at 50 mg/kg on day +3 after SCT. KRP203 (Novartis Pharma AG, Basel, Switzerland) dissolved in sterile water was orally administered at a dose of 1–3 mg/kg.

Evaluation of GVHD

Survival was monitored daily and clinical GVHD was assessed by using GVHD scoring system with five parameters [29]. For pathological analysis, tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Acute GVHD pathology was assessed using a semiquantitative scoring system in the small and large intestines, liver, and skin [30]. Gut pathological scores were sum of scores in the small intestine and colon. Pictures from tissue sections were taken at room temperature using a digital camera (DP72; Olympus, Tokyo, Japan) mounted on a microscope (BX51; Olympus).

Evaluation of GVL effects

Lethally irradiated B6D2F1 recipients were transplanted with purified T cells and T-cell depleted (TCD)-BM cells from B6 donors. Purification of T cells and TCD was performed using pan-T-cell Microbeads (Miltenyi Biotec, Auburn, CA) and anti-CD90-MicroBeads (Miltenyi Biotec), respectively, and the AutoMACS Pro Separator (Miltenyi Biotec) was used following the manufacturer's instructions. A total of 5×10^4 P815-luc$^+$ cells were injected to mice on day 0 of SCT. Following SCT, in vivo bioluminescence imaging (BLI) was conducted weekly to evaluate GVL effects. Mice were subcutaneously injected with 500 μg d-luciferin (Promega, Madison, WI), and in vivo imaging was done 5 min later. Luciferase$^+$ cells were detected using IVIS Imaging System ver. 4.3.1 (Perkin Elmer, Waltham, MA). Light emission is presented as photons per second per square centimeter per steer radiant (ph/s/cm^2/sr).

Flow cytometric analysis

Cell suspension was prepared from the liver, colon, and skin, as previously shown [31, 32]. Monoclonal antibodies (mAbs) used were FITC-, PE-, PECy7-, PerCP-, APC-, or APCCy7-conjugated anti-mouse CD4, CD8, CD45, CD45.1, TCRβ, H-2^d, and FoxP3 purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA), or Biolegend (San Diego, CA) (Supplementary Table 1). Apoptotic cells were stained with Annexin V-FITC. FoxP3 staining kit (eBioscience) was used for intracellular Foxp3 staining. Dead cells were determined based on the positivity of DAPI (Molecular Probes Inc., Eugene, OR). Cells were analyzed using a FACSCantoII (BD Bioscience, Tokyo, Japan) and FlowJo software (Tree Star, OR).

Cytometric beads array (CBA)

Plasma cytokine levels were determined using BD CBA Mouse Soluble Protein Flex Sets (BD Biosciences) and FACSCantoII (BD Bioscience).

Statistical analysis

Experiments were repeated at least twice to obtain eight or more samples for each group. Mann–Whitney *U* tests were used to compare data. Kaplan–Meier product limit method was used to obtain survival probability and the log-rank test was applied to compare survival curves. Cumulative incidence curve was used to obtain cumulative leukemia deaths and the Gray's test was applied to compare cumulative leukemia deaths curves. Analyses were performed using

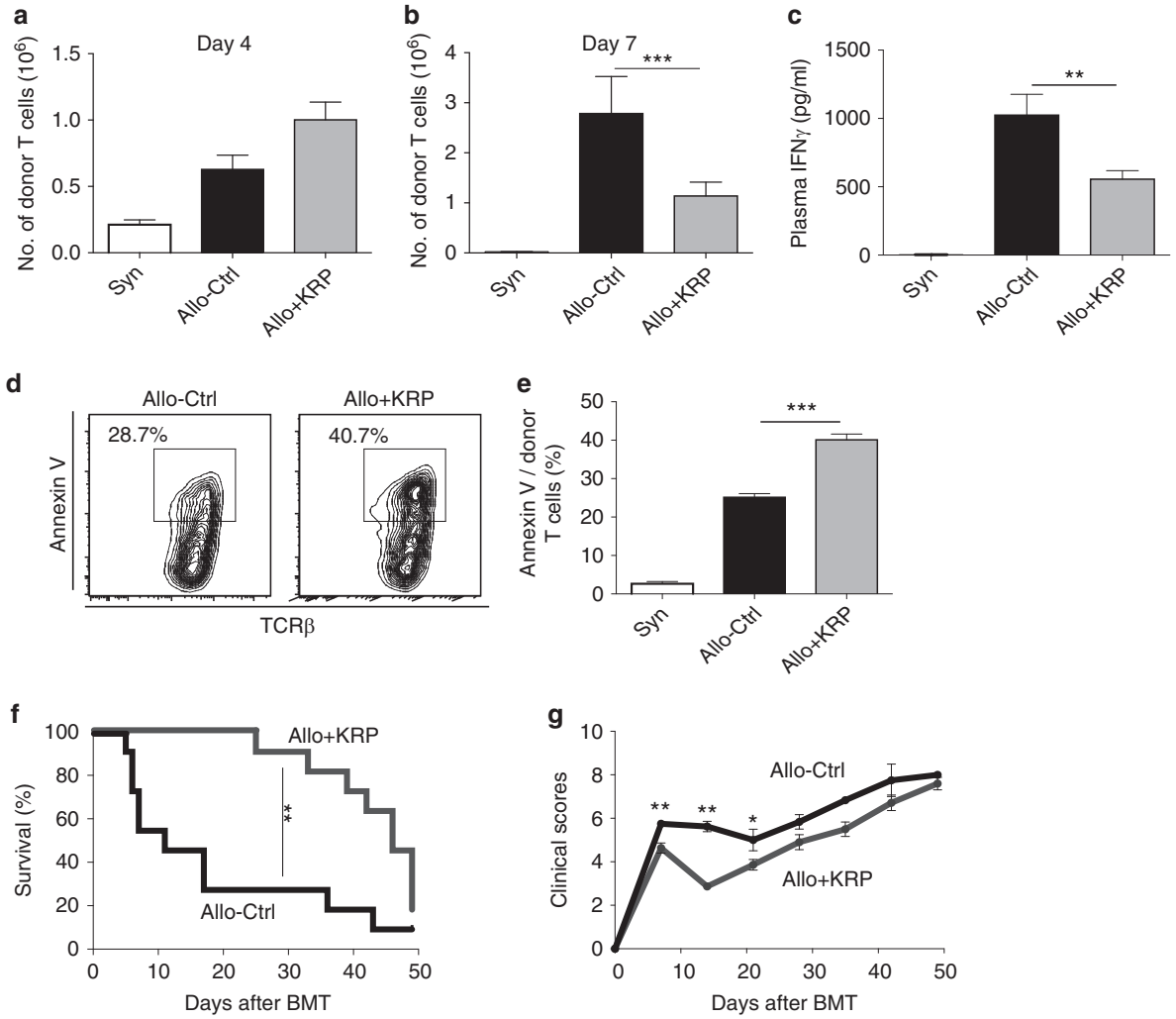

Fig. 1 KRP203 enhances apoptosis of donor T cells after allogeneic SCT and ameliorates GVHD. **a–e** Lethally irradiated B6D2F1 mice were transplanted with 10×10^6 splenocytes and 5×10^6 BM cells from MHC-haploidentical B6 mice. KRP203 at a dose of 1 mg/kg ($n = 8$) or diluent ($n = 8$) was orally administered daily from day 0. Syngeneic controls were B6 recipients transplanted from B6-Ly5a donors ($n = 3$). Absolute numbers of H-2K^{d}TCRβ^{+} donor T cells in allogeneic animals or CD45.1^{+}CD45.2^{-}TCRβ^{+} donor T cells in syngeneic controls were determined in the MLNs on day +4 (**a**) and day +7 **(b)** after SCT. Data from two independent experiments were combined and shown as mean ± SEM. **c** Plasma levels of IFN-γ on day +7 are shown as mean ± SEM ($n = 9$/group for allogeneic mice treated with diluent or KRP, and $n = 3$ for syngeneic controls). Cells harvested from the MLNs on day +6 posttransplant were stained with fluorescent-labeled Annexin V. Representative plots of Annexin V staining of donor T cells (**d**) and proportions of DAPI^{-} and Annexin V^{+} apoptotic cells among donor T cells (**e**). Data from two independent experiments are combined and shown as mean ± SEM ($n = 10$ for allogeneic mice treated with diluent or KRP and $n = 3$ for syngeneic control group). **f**, **g** SCT was performed as Fig. 1a. KRP203 (1 mg/kg, $n = 11$) or diluent ($n = 11$) was orally administered daily from day 0 to day +6. Survival (**f**) and clinical scores (**g**, mean ± SD)

GraphPad Prism 6 software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Short-term KRP203 induces earlier contraction of donor T cells in LNs and ameliorates GVHD

Lethally irradiated B6D2F1 (H-2$^{b/d}$, CD45.2^{+}) mice were transplanted with 5×10^6 bone marrow (BM) cells and 10×10^6 splenocytes from MHC-haploidentical B6 (H-2^{b}, CD45.2^{+}) donors on day 0. Syngeneic controls were B6 (H-2^{b}, CD45.2^{+}) mice transplanted with grafts from congenic B6-Ly5a (H-2^{b}, CD45.1^{+}) donors. KRP203 was orally administered at a dose of 1 mg/kg daily from day 0. Flow cytometric analysis of the mesenteric lymph nodes (MLNs) showed that donor T-cell expansion was significantly greater on both day +4 and day +7 after allogeneic SCT than that in syngeneic controls (Fig. 1a, b). There was a trend toward enhanced donor T-cell expansion on day 4 in KRP203-treated mice, while KRP203 induced earlier

contraction of a donor T-cell pool with significantly less levels of plasma IFN-γ on day +7 compared to allogeneic controls (Fig. 1c). We evaluated whether the KRP203-mediated earlier contraction of a donor T-cell pool on day +7 could be associated with an increase in donor T-cell apoptosis. Frequencies of $DAPI^-$ and Annexin V^+ apoptotic donor T cells on day +6 were significantly higher in the MLNs of KRP203-treated animals than those in allogeneic controls (Fig. 1d, e). These results suggest that KRP203 facilitates sequestration and apoptosis of donor T cells in the MLNs after allogeneic SCT, as has been shown in our previous study of FTY720 [24]. Administration of KRP203 from day 0 to day +6 significantly reduced morbidity and mortality of GVHD (Fig. 1f, g).

GVL effects were preserved in KRP203-treated recipients

Next, we tested impacts of KRP203 on graft-versus-leukemia (GVL) effects using in vivo BLI after MHC-haploidentical SCT. Lethally irradiated B6D2F1 mice were transplanted with 4×10^6 TCD-BM cells and 2×10^6 purified T cells from B6 donors together with 5×10^4 P815-luc$^+$ cells on day 0, followed by oral administration of KRP203 at a dose of 3 mg/kg daily from day 0 to day +28. In controls, cyclosporin (CSP) was orally administered daily during the same period at a dose of 50 mg/kg, as previously described [16, 33]. In vivo BLI performed weekly after SCT demonstrated that all mice receiving TCD-BM alone died with massive proliferation of P815-luc$^+$. Although allogeneic mice mounted potent GVL effects, all the mice succumbed to severe GVHD without any evidence of tumor growth (Fig. 2a–c). Remarkably, GVL effect was much potent in the KRP203-treated animals than that in the CSP-treated mice (Fig. 2b), resulted in significantly improved overall survival (Fig. 2c).

A combination of short-term KRP203 and PTCY is superior to PTCY alone in ameliorating GVHD

Next, we evaluated whether addition of KRP203 could reduce GVHD in combination with PTCY. We found that cyclophosphamide (CY) i.p. injected at a dose of 100 mg/kg on day +3 significantly reduced GVHD mortality than 50 mg/kg of CY (Fig. 3a). In the following experiments, we utilized CY at a dose of 50 mg/kg to mimic clinical HLA-haploidentical SCT, where PTCY alone was not sufficient to prevent GVHD [3, 34].

We then evaluated whether addition of short-term (day +0 to day +4) or long-term administration (day +0 to day +28) of KRP203 could improve effects of PTCY. Short-term administration early after SCT was enough to ameliorate GVHD in combination with PTCY (Supplementary Fig. 1). Although GVHD was severe in allogeneic controls

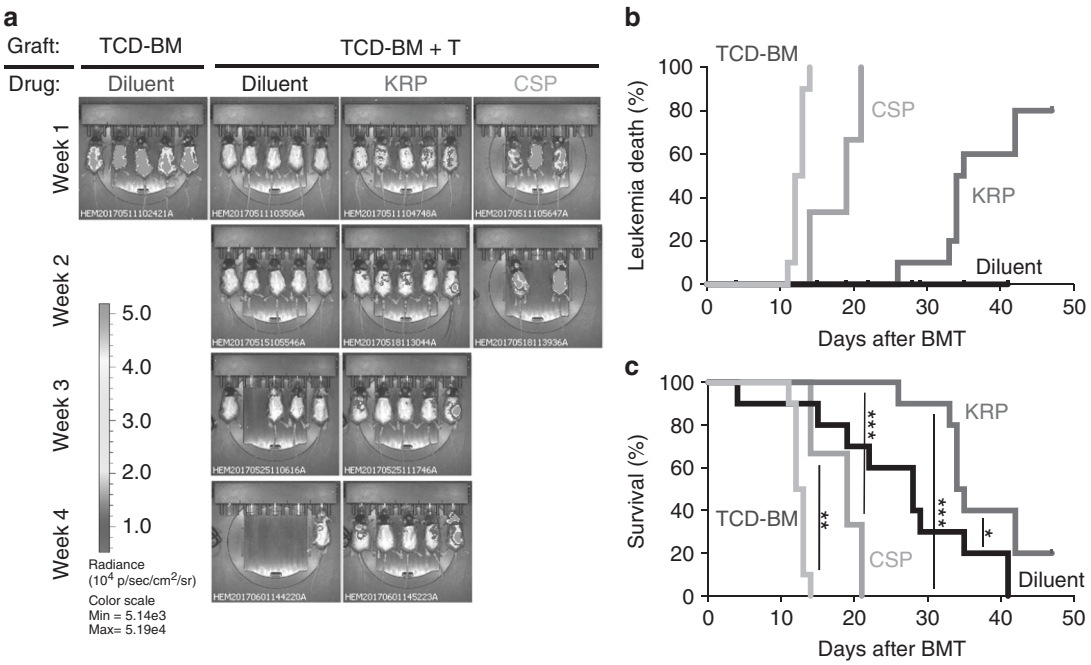

Fig. 2 A combination of short-term KRP203 and PTCY spares significant GVL effects after allogeneic SCT. **a–c** Lethally irradiated B6D2F1 mice were transplanted with 4×10^6 TCD-BM with or without 2×10^6 T cells from B6 donors, together with 5×10^4 P815-luc cells on day 0. KRP203 (3 mg/kg) or CSP (50 mg/kg) was orally administered from day 0 to day +28. Tumor growth in the recipients was weekly monitored in vivo BLI. Representative images of BLI (**a**), cumulative leukemia death (**b**), and survival curves (**c**) after SCT are shown (TCD-BM, $n = 10$; diluent, $n = 10$, KRP, $n = 10$; CSP, $n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

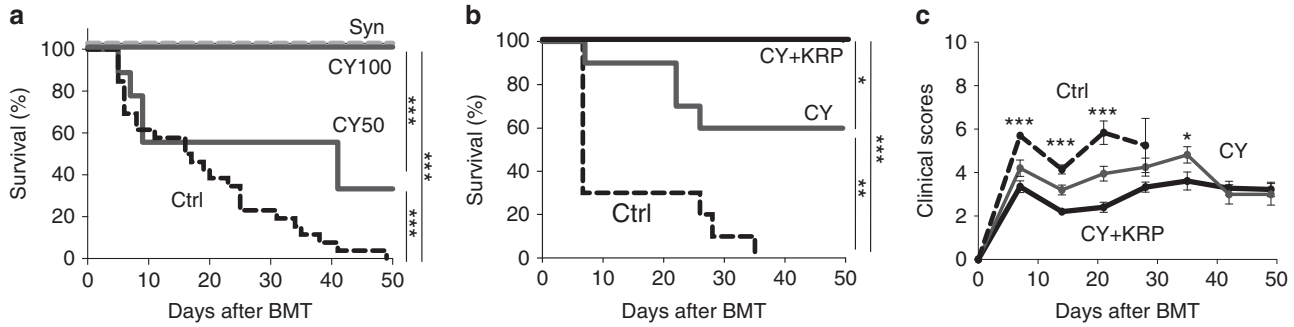

Fig. 3 Short-term KRP203 synergistically suppresses GVHD with PTCY. Lethally irradiated B6D2F1 mice were transplanted with 10×10^6 splenocytes and 5×10^6 BM cells from B6D2F1 (syngeneic) or B6 (allogeneic) donors. **a** Allogeneic mice were i.p. injected with CY at a dose of 50 mg/kg (CY50, $n = 9$) or 100 mg/kg (CY100, $n = 26$), or diluent (Ctrl, $n = 26$) on day +3. Survivals of these recipients were shown with those of syngeneic mice (Syn, $n = 2$). Allogeneic mice were i.p. injected with CY (50 mg/kg) on day +3 in combination with oral injection of KRP203 (1 mg/kg, CY+KRP) or diluent (CY) on days 0 to +4. Survivals (**b**) and clinical GVHD scores (**c**, mean ± SD) of these mice were shown. Data from two experiments were combined ($n = 10$/group)

with 100% mortality by day +30 after SCT, CY alone again significantly reduced morbidity and mortality of GVHD (Fig. 3b, c). A combination of CY and KRP203 further reduced morbidity and mortality of GVHD compared to CY alone (Fig. 3b, c). We also evaluated GVHD pathology of the gut, liver, and skin 4 weeks after SCT. Pathology of allogeneic animals showed crypt apoptosis with mononuclear cell (MNC) infiltration in the gut, MNC infiltration to the portal triads and bile ducts accompanied by coagulative necrosis of hepatocytes in the liver, and MNC infiltration with loss of fat layer in the skin (Fig. 4a). PTCY alone significantly reduced GVHD pathology scores in the gut, liver, and skin (Fig. 4b–d). A combination of PTCY and KRP203 further reduced GVHD pathological scores in the gut (Fig. 4b). Plasma levels of TNF-α on day +14 were significantly less in mice treated with a combination of PTCY and KRP203 than those in allogeneic mice receiving CY alone (Fig. 5a). Donor T-cell infiltration was assessed by flow cytometric analysis of the gut, liver, and skin on day +15. PTCY alone significantly suppressed donor T-cell infiltration to each organ (Fig. 5b–d). Addition of KRP203 to PTCY further reduced donor T-cell infiltration in the colon and skin (Fig. 5b–d).

Short-term KRP203 in combination with PTCY improved Treg reconstitution after SCT

It has been shown that Tregs play a critical role in PTCY-mediated GVHD suppression [11, 13, 35]. On the other hand, CNIs disturb persistence and reconstitution of Tregs through inhibition of IL-2 [16, 17]. Administration of CY at a dose of 50 mg/kg on day +3 significantly enhanced reconstitution of donor $CD4^+FoxP3^+$ Tregs in the spleen on day +21 compared to allogeneic controls and CSP-treated mice (Supplementary Fig. 2). A combination of PTCY and KRP203 significantly increased $CD4^+$ $FoxP3^+$ Tregs in the spleen on day +21 compared to PTCY alone (Fig. 5e).

Discussion

Emerging evidences indicate that PTCY is a safe and effective GVHD prophylaxis and has increasingly been used in HLA-haploidentical SCT [36]. A recent clinical study suggested that PTCY-based GVHD prophylaxis results in better GVHD control than standard CNI-based GVHD prophylaxis in HLA identical SCT, and this has been tested in a prospective randomized study [37]. However, PTCY alone is not sufficient to efficiently prevent GVHD and required additional immunosuppressants such as CNIs [14, 38]. Tregs play an important role in tolerance induction after SCT [11–13]. Tregs increase their expression of aldehyde dehydrogenase 2 (ALDH2) after allogeneic SCT and acquire resistance against PTCY-induced apoptosis [11, 35]. Because CNIs negatively affect Treg function by inhibiting IL-2 signaling, addition of CNIs to PTCY possibly hampers the establishment of immune tolerance after SCT [16]. To avoid these adverse effects of CNIs, CNI-free PTCY-based GVHD prophylaxis using sirolimus or bortezomib is under development [19, 39, 40]. In the current study, we found that a novel CNI-free GVHD prophylaxis, short-term KRP203 in combination with PTCY reduced donor T-cell infiltration in the gut and skin, enhanced Treg reconstitution, and ameliorated GVHD after allogeneic SCT compared to PTCY alone.

S1PR modulator is a new class of immunosuppressants; a first-in-class agent FTY720 having a high affinity for S1PR1,3,4,5 has been approved by FDA for treatment of multiple sclerosis [41, 42]. FTY720 mitigates harmful immune responses largely but not exclusively by sequestrating T cells in the SLOs [21, 22]. We and others have

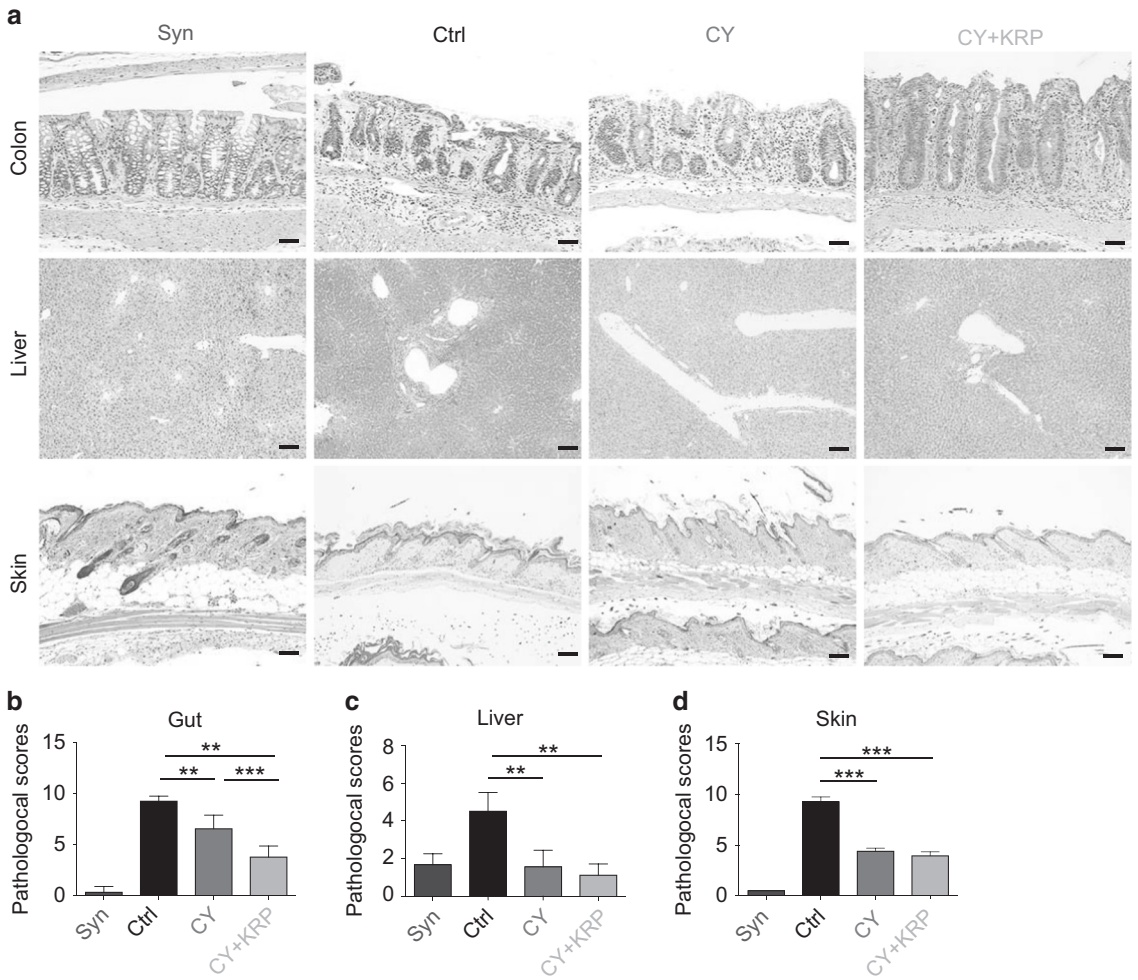

Fig. 4 Short-term KRP203 synergistically suppresses gut pathological GVHD with PTCY. Mice were transplanted and treated with CY alone or CY plus KRP203 as in Fig. 3b. **a–c** Pathological GVHD were evaluated in the colon, liver, and skin 4 weeks after SCT. **a** Representative images of H&E. Pathological GVHD scores of gut (**b**), liver (**c**), and skin (**d**) from two experiments were combined and shown as mean ± SEM (Syn, $n = 3$; Ctrl, $n = 4$; CY, $n = 9$; CY+KRP, $n = 9$)

previously shown that FTY720-induced apoptosis of donor T cells without interfering Treg function, resulting in GVHD mitigation [23, 24, 43, 44]. KRP203 acts specifically on $S1PR_1$ with a potentially milder toxicity profile. Vascular endothelial cells express both $S1PR_1$ and $S1PR_3$ [45]. FTY720 antagonizes $S1PR_1$ and $S1PR_3$ on vascular endothelial cells and increases vascular permeability, which is associated with FTY720-induced adverse effects such as macular edema [46]. KRP203 spares $S1PR_3$ signaling in vascular endothelial cells and thus potentially induces less vascular adverse events compared to FTY720. Thus, KRP203 may be a promising agent to serve as a substitute of CNIs that is used in combination with PTCY [27, 28]. As expected, donor T cells in the skin and gut were significantly less in mice treated with PTCY+KRP203 than in those treated with PTCY alone, leading to reduced pathological GVHD scores in the gut. The mechanism by which KRP203 enhanced reduction of donor T cells in the GVHD target organs remains to be elucidated. KRP203 enhanced activation of donor alloreactive T cells by sequestrating donor T cells within the lymph nodes and made them more susceptible to PTCY-induced cell death. Kataoka et al. reported that FTY720 started from day 0, but not started from day +2, mitigated mouse GVHD, suggesting that the enhancement of donor T-cell trapping within the SLOs immediately after SCT is critical for GVHD prophylaxis by S1PR modulator [47]. In addition, S1PR modulator exerts pro-apoptotic effects in the SLOs [48]. We confirmed that KRP203 administrated only on day 0 to +4 was sufficient to enhance GVHD prophylaxis of PTCY, suggesting that this synergistic effects are not solely dependent on T-cell sequestration [49].

In contrast to the negative impact of CNIs on Treg reconstitution, KRP203 enhanced Treg expansion when added to PTCY. Previous studies showed that S1P agonist differentially affected the homing properties of Tregs

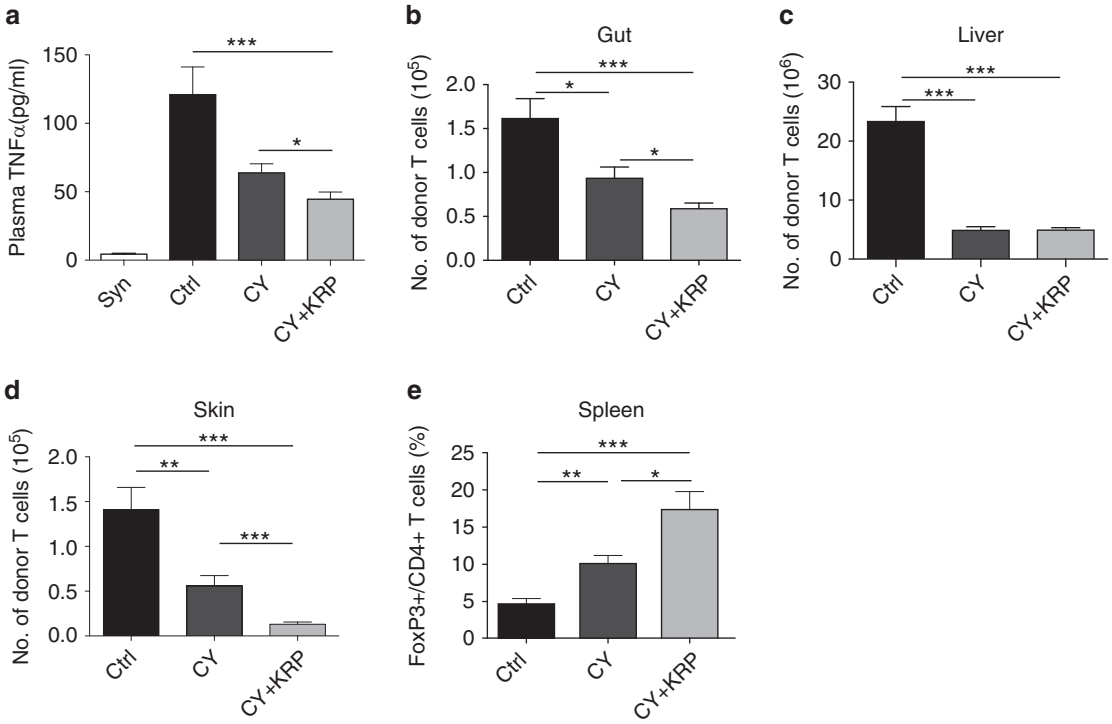

Fig. 5 Short-term KRP203 synergistically suppresses donor T-cell infiltration in the gut and skin with PTCY. **a** Plasma levels of TNF-α on day +14 post-SCT from three experiments were combined and shown as mean ± SEM (Syn, $n = 3$; Ctrl, $n = 14$; CY, $n = 13$; CY+KRP, $n = 14$). **b–d** Absolute numbers of donor T cells in the colons (**b**), livers (**c**), and skins (**d**) from allogeneic mice treated with diluent ($n = 7$), CY ($n = 7$), or CY and KRP203 ($n = 8$) were determined using flow cytometric analysis on day +15. **e** Proportion of donor Tregs among $CD4^+$ donor T cells in the spleen were determined on day +21 post-SCT (Ctrl, $n = 9$; CY, $n = 8$; CY+KRP, $n = 8$). Data from two similar experiments were combined and shown as mean ± SEM. Scale bars: colon, 50 μm; liver and skin, 100 μm. $*p < 0.05$, $**p < 0.01$; $***p < 0.005$

compared to other T-cell subsets due to lower expression of $S1PR_1$ on Tregs. The less Treg homing to the SLOs compared to conventional T cells after KRP203-treatment might lead to less apoptosis in Tregs compared to conventional T cells, resulted in more Treg persistence in KRP203-treated mice [50]. FTY720 converts antigen-stimulated conventional T cells to $FoxP3^+$ T cells in vitro [51]. Although the mechanism by which KRP203 enhanced Treg expansion remained to be clarified, KRP203 is a reasonable candidate to be combined with PTCY for GVHD prophylaxis, in which Tregs could play a critical role for GVHD prophylaxis [13].

We found that GVL effect was slightly impaired but not abrogated by KRP203, while CSP profoundly attenuated GVL effects. Our results were consistent with previous reports showing that long-term FTY720 ameliorates GVHD with reduced but persisting GVL effect [23, 44]. Thus, combination of PTCY with KRP203 may have an advantage to maintain GVL effect over a combination of PTCY and CNIs.

Acknowledgements This study was supported by JSPS KAKENHI (17K19641 and 17H04206 to TT, 17K09945 to DH), Japan Society of Hematology Research Fund (TT), the Center of Innovation Program from JST (TT), and research grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research (DH).

Compliance with ethical standards

Conflict of interest TT is a consultant of Novartis Pharma AG and received a research grant from Novartis Pharma AG.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using post-transplantation cyclophosphamide. Biol Blood Marrow Transplant. 2002;8:377–86.
2. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. Biol Blood Marrow Transplant. 2008;14:641–50.
3. Luznik L, Bolanos-Meade J, Zahurak M, Chen AR, Smith BD, Brodsky R, et al. High-dose cyclophosphamide as single-agent,

short-course prophylaxis of graft-versus-host disease. Blood. 2010;115:3224–30. https://doi.org/10.1182/blood-2009-11-251595.
4. Brunstein CG, Fuchs EJ, Carter SL, Karanes C, Costa LJ, Wu J, et al. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. Blood. 2011;118:282–8. https://doi.org/10.1182/blood-2011-03-344853.
5. Bashey A, Zhang X, Sizemore CA, Manion K, Brown S, Holland HK, et al. T-cell-replete HLA-haploidentical hematopoietic transplantation for hematologic malignancies using post-transplantation cyclophosphamide results in outcomes equivalent to those of contemporaneous HLA-matched related and unrelated donor transplantation. J Clin Oncol. 2013;31:1310–6. https://doi.org/10.1200/JCO.2012.44.3523.
6. Sugita J, Kawashima N, Fujisaki T, Kakihana K, Ota S, Matsuo K, et al. HLA-haploidentical peripheral blood stem cell transplantation with post-transplant cyclophosphamide after busulfan-containing reduced-intensity conditioning. Biol Blood Marrow Transplant. 2015;21:1646–52. https://doi.org/10.1016/j.bbmt.2015.06.008.
7. Kanakry CG, Bolanos-Meade J, Kasamon YL, Zahurak M, Durakovic N, Furlong T, et al. Low immunosuppressive burden after HLA-matched related or unrelated BMT using posttransplantation cyclophosphamide. Blood. 2017;129:1389–93. https://doi.org/10.1182/blood-2016-09-737825.
8. Berenbaum MC, Brown IN. Prolongation of homograft survival in mice with single doses of cyclophosphamide. Nature. 1963; 200:84.
9. Mayumi H, Himeno K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. VI. Tolerance induction in H-2-haplotype-identical strain combinations in mice. Transplantation. 1985;40:188–94. e-pub ahead of print 1985/08/01.
10. Mayumi H, Good RA. Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. J Exp Med. 1989;169: 213–38.
11. Kanakry CG, Ganguly S, Zahurak M, Bolanos-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. Sci Transl Med. 2013;5:211ra157 https://doi.org/10.1126/scitranslmed.3006960.
12. Kanakry CG, Coffey DG, Towlerton AM, Vulic A, Storer BE, Chou J, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. JCI Insight. 2016;1; https://doi.org/10.1172/jci.insight.86252.
13. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. J Clin Investig. 2019;130:2357–73. https://doi.org/10.1172/JCI124218. e-pub ahead of print 2019/03/27.
14. Ruggeri A, Labopin M, Bacigalupo A, Afanasyev B, Cornelissen JJ, Elmaagacli A, et al. Post-transplant cyclophosphamide for graft-versus-host disease prophylaxis in HLA matched sibling or matched unrelated donor transplant for patients with acute leukemia, on behalf of ALWP-EBMT. J Hematol Oncol. 2018;11:40 https://doi.org/10.1186/s13045-018-0586-4.
15. Hingorani S. Renal complications of hematopoietic-cell transplantation. N Engl J Med. 2016;374:2256–67. https://doi.org/10.1056/NEJMra1404711.
16. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. Blood. 2006;108: 390–9. https://doi.org/10.1182/blood-2006-01-0329.
17. Sugiyama H, Maeda Y, Nishimori H, Yamasuji Y, Matsuoka K, Fujii N, et al. Mammalian target of rapamycin inhibitors permit regulatory T cell reconstitution and inhibit experimental chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2014;20:183–91. https://doi.org/10.1016/j.bbmt.2013.11.018.
18. Rosen H, Gonzalez-Cabrera PJ, Sanna MG, Brown S. Sphingosine 1-phosphate receptor signaling. Annu Rev Biochem. 2009; 78:743–68. https://doi.org/10.1146/annurev.biochem.78.072407.103733.
19. Fujita T, Hirose R, Yoneta M, Sasaki S, Inoue K, Kiuchi M, et al. Potent immunosuppressants, 2-alkyl-2-aminopropane-1,3-diols. J Med Chem. 1996;39:4451–9. https://doi.org/10.1021/jm9603911.
20. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem. 2002;277:21453–7.
21. Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science. 2002;296:346–9. https://doi.org/10.1126/science.1070238.
22. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature. 2004;427:355–60. https://doi.org/10.1038/nature02284.
23. Kim YM, Sachs T, Asavaroengchai W, Bronson R, Sykes M. Graft-versus-host disease can be separated from graft-versus-lymphoma effects by control of lymphocyte trafficking with FTY720. J Clin Investig. 2003;111:659–69. https://doi.org/10.1172/JCI16950.
24. Hashimoto D, Asakura S, Matsuoka K, Sakoda Y, Koyama M, Aoyama K, et al. FTY720 enhances the activation-induced apoptosis of donor T cells and modulates graft-versus-host disease. Eur J Immunol. 2007;37:271–81. https://doi.org/10.1002/eji.200636123.
25. Tedesco-Silva H, Szakaly P, Shoker A, Sommerer C, Yoshimura N, Schena FP, et al. FTY720 versus mycophenolate mofetil in de novo renal transplantation: six-month results of a double-blind study. Transplantation. 2007;84:885–92. https://doi.org/10.1097/01.tp.0000281385.26500.3b.
26. Rosen H, Stevens RC, Hanson M, Roberts E, Oldstone MB. Sphingosine-1-phosphate and its receptors: structure, signaling, and influence. Annu Rev Biochem. 2013;82:637–62. https://doi.org/10.1146/annurev-biochem-062411-130916. e-pub ahead of print 2013/03/27.
27. Shimizu H, Takahashi M, Kaneko T, Murakami T, Hakamata Y, Kudou S, et al. KRP-203, a novel synthetic immunosuppressant, prolongs graft survival and attenuates chronic rejection in rat skin and heart allografts. Circulation. 2005;111:222–9. https://doi.org/10.1161/01.CIR.0000152101.41037.AB.
28. Khattar M, Deng R, Kahan BD, Schroder PM, Phan T, Rutzky LP, et al. Novel sphingosine-1-phosphate receptor modulator KRP203 combined with locally delivered regulatory T cells induces permanent acceptance of pancreatic islet allografts. Transplantation. 2013;95:919–27. https://doi.org/10.1097/TP.0b013e3182842396.
29. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J Jr., Crawford JM, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. Blood. 1996;88:3230–9.
30. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med. 2002;8:575–81. https://doi.org/10.1038/nm0602-575.
31. Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, et al. Origin of the lamina propria dendritic cell network. Immunity. 2009;31:513–25. https://doi.org/10.1016/j.immuni.2009.08.010.

32. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103+ DCs. J Exp Med. 2009;206:3115–30. https://doi.org/10.1084/jem.20091756.
33. Fidler JM, Ku GY, Piazza D, Xu R, Jin R, Chen Z. Immunosuppressive activity of the Chinese medicinal plant Tripterygium wilfordii. III. Suppression of graft-versus-host disease in murine allogeneic bone marrow transplantation by the PG27 extract. Transplantation. 2002;74:445–57.
34. Kanakry CG, Tsai HL, Bolanos-Meade J, Smith BD, Gojo I, Kanakry JA, et al. Single-agent GVHD prophylaxis with posttransplantation cyclophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. Blood. 2014;124:3817–27. https://doi.org/10.1182/blood-2014-07-587477.
35. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor CD4+ Foxp3+ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. Blood. 2014;124:2131–41. https://doi.org/10.1182/blood-2013-10-525873.
36. Apperley J, Niederwieser D, Huang XJ, Nagler A, Fuchs E, Szer J, et al. Haploidentical hematopoietic stem cell transplantation: a global overview comparing Asia, the European Union, and the United States. Biol Blood Marrow Transplant. 2016;22:23–26. https://doi.org/10.1016/j.bbmt.2015.11.001.
37. Bolanos-Meade J, Reshef R, Fraser R, Fei M, Abhyankar S, Al-Kadhimi Z, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). Lancet Haematol. 2019;6:e132–e143. https://doi.org/10.1016/S2352-3026(18)30221-7.
38. Alousi AM, Brammer JE, Saliba RM, Andersson B, Popat U, Hosing C, et al. Phase II trial of graft-versus-host disease prophylaxis with post-transplantation cyclophosphamide after reduced-intensity busulfan/fludarabine conditioning for hematological malignancies. Biol Blood Marrow Transplant. 2015;21:906–12. https://doi.org/10.1016/j.bbmt.2015.01.026.
39. Cieri N, Greco R, Crucitti L, Morelli M, Giglio F, Levati G, et al. Post-transplantation cyclophosphamide and sirolimus after haploidentical hematopoietic stem cell transplantation using a treosulfan-based myeloablative conditioning and peripheral blood stem cells. Biol Blood Marrow Transplant. 2015;21:1506–14. https://doi.org/10.1016/j.bbmt.2015.04.025.
40. Al-Homsi AS, Cole K, Bogema M, Duffner U, Williams S, Mageed A. Short course of post-transplantation cyclophosphamide and bortezomib for graft-versus-host disease prevention after allogeneic peripheral blood stem cell transplantation is feasible and yields favorable results: a phase I study. Biol Blood Marrow Transplant. 2015;21:1315–20. https://doi.org/10.1016/j.bbmt.2015.02.008.
41. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Annu Rev Immunol. 2012;30:69–94. https://doi.org/10.1146/annurev-immunol-020711-075011.
42. Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med. 2010;362:402–15. https://doi.org/10.1056/NEJMoa0907839.
43. Lee RS, Kuhr CS, Sale GE, Zellmer E, Hogan WJ, Storb R, et al. FTY720 does not abrogate acute graft-versus-host disease in the dog leukocyte antigen-nonidentical unrelated canine model. Transplantation. 2003;76:1155–8. https://doi.org/10.1097/01.TP.0000083891.14089.B8.
44. Taylor PA, Ehrhardt MJ, Lees CJ, Tolar J, Weigel BJ, Panoskaltsis-Mortari A, et al. Insights into the mechanism of FTY720 and compatibility with regulatory T cells for the inhibition of graft-versus-host disease (GVHD). Blood. 2007;110:3480–8. https://doi.org/10.1182/blood-2007-05-087940.
45. Hla T. Physiological and pathological actions of sphingosine 1-phosphate. Semin Cell Dev Biol. 2004;15:513–20. https://doi.org/10.1016/j.semcdb.2004.05.002.
46. Blaho VA, Hla T. An update on the biology of sphingosine 1-phosphate receptors. J Lipid Res. 2014;55:1596–608. https://doi.org/10.1194/jlr.R046300.
47. Kataoka H, Ohtsuki M, Shimano K, Mochizuki S, Oshita K, Murata M, et al. Immunosuppressive activity of FTY720, sphingosine 1-phosphate receptor agonist: II. Effect of FTY720 and FTY720-phosphate on host-versus-graft and graft-versus-host reaction in mice. Transpl Proc. 2005;37:107–9. https://doi.org/10.1016/j.transproceed.2004.12.287.
48. White C, Alshaker H, Cooper C, Winkler M, Pchejetski D. The emerging role of FTY720 (Fingolimod) in cancer treatment. Oncotarget. 2016;7:23106–27. https://doi.org/10.18632/oncotarget.7145.
49. Pinschewer DD, Ochsenbein AF, Odermatt B, Brinkmann V, Hengartner H, Zinkernagel RM. FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. J Immunol. 2000;164:5761–70. e-pub ahead of print 2000/05/23.
50. Sawicka E, Dubois G, Jarai G, Edwards M, Thomas M, Nicholls A, et al. The sphingosine 1-phosphate receptor agonist FTY720 differentially affects the sequestration of CD4+/CD25+ T-regulatory cells and enhances their functional activity. J Immunol. 2005;175:7973–80.
51. Sehrawat S, Rouse BT. Anti-inflammatory effects of FTY720 against viral-induced immunopathology: role of drug-induced conversion of T cells to become Foxp3+ regulators. J Immunol. 2008;180:7636–47. https://doi.org/10.4049/jimmunol.180.11.7636. e-pub ahead of print 2008/05/21.