REGULAR ARTICLE

blood advances

Phosphorylated ERK1/2 in CD4 T cells is associated with acute GVHD in allogeneic hematopoietic stem cell transplantation

Hidekazu Itamura,[1] Takero Shindo,[2] Satoshi Yoshioka,[3] Takayuki Ishikawa,[3] and Shinya Kimura[1]

[1]Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, Japan; [2]Department of Hematology/Oncology, Kyoto University Graduate School of Medicine, Kyoto, Japan; and [3]Department of Hematology, Kobe City Medical Center General Hospital, Kobe, Japan

Key Points

- Phospho-ERK1/2 levels in peripheral blood CD4$^+$ T cells at day 30 after allo-HSCT may be a biomarker of human acute GVHD.
- Our results provide a rationale for the efficacy of MEK inhibitors against human GVHD.

To diagnose graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT) is sometimes difficult. We showed previously that MEK inhibitors selectively suppress murine GVHD while retaining antiviral and antitumor immunity. Here, we asked whether the RAS/MEK/ERK pathway is activated in human allo-HSCT recipients with GVHD, and whether the phosphorylated ERK1/2 can be a biomarker of GVHD. Peripheral blood was sequentially collected from 20 allo-HSCT recipients: 1 bone marrow transplant, 7 peripheral blood stem cell transplants (PBSCT), and 12 cord blood transplants. Ten of the 20 allo-HSCT recipients developed GVHD, and phosphorylation of ERK1/2 in T and B cells was analyzed by flow cytometry. Occurrence of acute GVHD was associated with phosphorylation of ERK1/2 in CD4$^+$ T cells at day 30 ($P < .001$), which was suppressed by ex vivo exposure to a MEK inhibitor trametinib at clinically achievable concentrations. In particular, ERK1/2 was phosphorylated preferentially in naive/central memory CD4$^+$ T cells. Notably, phosphorylation of ERK1/2 fell as GVHD improved. These results suggest that phosphorylation status of ERK1/2 in peripheral blood CD4$^+$ T cells may be a future biomarker for diagnosing human GVHD, and the potential efficacy of MEK inhibitors against human GVHD.

Introduction

After allogeneic hematopoietic stem cell transplantation (allo-HSCT), donor-derived T cells trigger host tissue injury; that is, graft-versus-host disease (GVHD).[1-3] Although currently available immunosuppressants reduce GVHD, they also suppress antiviral/antitumor T cells, which can result in fatal opportunistic infections[4] and relapse of hematologic malignancy.[5]

Recently, we showed that MEK inhibitors selectively suppress naive and central memory T cells in vitro while preserving the activity of effector memory T cells.[6] We then confirmed that a MEK inhibitor trametinib selectively suppresses GVHD while retaining antitumor immunity in vivo,[7] and that it also attenuates delayed rejection sparing thymic function after lung transplantation.[8] Although these findings should be verified in clinical trials, the importance of the MEK/ERK pathway in human GVHD is still unclear.

The diagnosis of GVHD mainly depends on pathological evaluation. However, correct diagnosis is often difficult because of the unavailability of fair and sufficient specimens. As currently available laboratory tests have limitations, more specific biomarkers of GVHD are needed.

Here, we asked whether the RAS/MEK/ERK pathway is activated in T cells of patients with GVHD. In addition, we asked whether phosphorylation status of ERK1/2 in peripheral blood T cells can be

Submitted 22 April 2019; accepted 15 January 2020; published online 20 February 2020. DOI 10.1182/bloodadvances.2019000343.

All inquiry e-mails about the data should be sent to the corresponding author, Takero Shindo (takeros@kuhp.kyoto-u.ac.jp).

The full-text version of this article contains a data supplement.

a predictive and diagnostic biomarker for GVHD by examining the association between phosphorylated ERK1/2 levels in T cells and the occurrence of GVHD. Finally, we exposed T cells to trametinib ex vivo to estimate the dose required to suppress GVHD.

Study design

Twenty allo-HSCT recipients at Saga University Hospital and Kobe City Medical Center General Hospital from May 2016 through May 2017 were enrolled. Their clinical characteristics are summarized in Table 1. All patients achieved complete chimerism with donor type. Peripheral blood mononuclear cells (PBMCs) were collected sequentially on days 30, 60, and 90 post–allo-HSCT. The used antibodies and reagents are described in supplemental Materials. Trametinib and tacrolimus were purchased from Selleck Chemicals (Houston, TX), and detailed protocols of in vitro and ex vivo experiments are described in supplemental Materials. The study was approved by the Institutional Review Boards of Saga University Hospital and Kobe City Medical Center General Hospital.

Results and discussion

The clinical characteristics of the patients are summarized in Table 1 and supplemental Materials. Ten patients (50%) developed acute GVHD at days 13 to 34 (median, day 24); 5 (50%) of these developed grade III to IV GVHD. One patient with acute GVHD later developed chronic GVHD. Patients with acute GVHD showed slightly delayed reconstitution of lymphocytes at days 30 and 90 (supplemental Figure 1A-B). Five patients with acute GVHD had viral infectious episodes: 4 cases after the development of GVHD and 1 case simultaneously with GVHD, which may be associated with delayed immune reconstitution (supplemental Table 1).

To evaluate the potential role of the RAS/MEK/ERK signaling pathway in GVHD, we analyzed phosphorylation status of ERK1/2 in peripheral blood T and B cells stimulated with or without phorbol 12-myristate 13-acetat (PMA) and ionomycin. Notably, phospho-ERK1/2 levels at day 30 were higher, particularly in $CD4^+$ T cells from patients with acute GVHD than in patients without GVHD ($P < .001$; Figure 1A-B). In contrast, phospho-ERK1/2 levels in $CD8^+$ T and $CD19^+$ B cells from patients with GVHD were equivalent to those in patients without GVHD (Figure 1C-D). Phospho-ERK1/2 levels at day 30 were higher in $CD4^+$-naive ($CD27^+CD45RA^+$) and central memory ($CD27^+CD45RA^-$) T cells in patients with acute GVHD than in patients without GVHD ($P = .003$ and $P = .022$; Figure 1E-F). There was no difference in phospho-ERK1/2 levels in effector memory T cells ($CD27^-CD45RA^-$) between patients with GVHD and patients without GVHD (Figure 1G). Furthermore, we found that phospho-ERK1/2 levels were associated with clinical course. One patient who developed grade III acute GVHD at day 30 received prednisolone pulse therapy; GVHD improved by day 60 and coincided with decreased expression of phospho-ERK1/2 (Figure 1H).

To investigate whether trametinib suppresses activation of RAS/MEK/ERK signaling in $CD4^+$ and $CD8^+$ T cells of the patients, PBMCs of the patients with GVHD at day 30 were stimulated ex vivo with PMA/ionomycin and trametinib or tacrolimus at differential concentrations (Figure 2A-B). Trametinib strongly suppressed phosphorylation of ERK1/2 in $CD4^+$ T, $CD8^+$ T, and $CD19^+$ B cells in a dose-dependent manner, whereas tacrolimus did not. Based on pharmacokinetics of trametinib in patients with melanoma,[9] our results indicate that its clinically achievable concentrations suppress activation of the MEK/ERK pathway in T and B cells of patients with GVHD.

Table 1. Patient characteristics

Characteristics	GVHD-positive	GVHD-negative	P
Total number	10	10	
Male, n (%)	8 (80)	6 (60)	.3553
Age, median (range), y	55 (20-62)	50 (21-66)	.5620
Onset of acute GVHD, median (range), d	24 (13-34)		
Acute GVHD grade, n (%)			
I	2 (20)		
II	3 (30)		
III-IV	5 (50)		
Onset of chronic GVHD, n (%)	1 (10)	0 (0)	
WBC count, mean, $\times 10^9$/L			
Day 30	3.217	3.134	.9143
Day 60	5.909	5.927	.9917
Day 90	6.355	5.242	.5381
Lymphocyte count, mean, $\times 10^9$/L			
Day 30	0.330	0.602	.0858
Day 60	0.881	1.159	.6095
Day 90	0.830	1.426	.0911
Disease, n (%)			
AML/MDS	6 (60)	5 (50)	
ALL	0 (0)	2 (20)	
ML/ATLL	2 (20)	1 (10)	
Other	2 (20)	2 (20)	
Donor source			
BMT	0 (0)	1 (10)	
PBSCT	2 (20)	4 (40)	
CBT	8 (80)	4 (40)	
Haplo	0 (0)	1 (10)	
Conditioning, n (%)			
MAC	3 (30)	3 (30)	
RIST	7 (70)	7 (70)	
Immunosuppressant, n (%)			
FK base	10 (100)	9 (90)	
CyA base	0 (0)	1 (10)	
+ATG	0 (0)	4 (40)	
+PT-CY	0 (0)	1 (10)	
Relapse after HSCT	1 (10)	3 (30)	

ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; ATLL, adult T-cell leukemia/lymphoma; BMT, bone marrow transplantation; CBT, cord blood transplantation; CyA, cyclosporine; FK, tacrolimus; Haplo, haploidentical transplantation; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; ML, malignant lymphoma; PBSCT, peripheral blood stem cell transplantation; RIST, reduced-intensity hematopoietic stem cell transplantation; PT-CY, post-transplant cyclophosphamide.

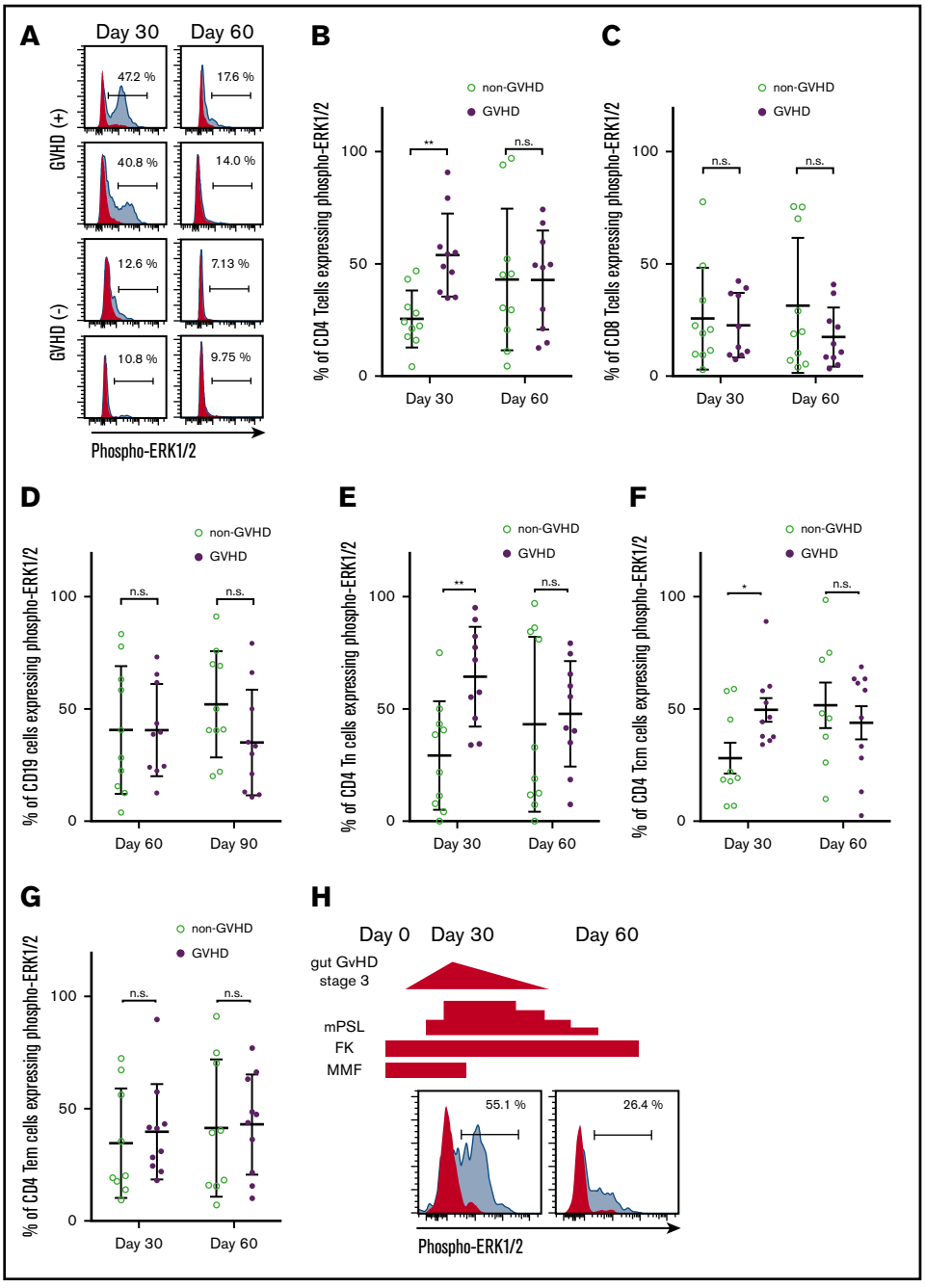

Figure 1. Phospho-ERK1/2 levels in CD4$^+$-naive and central memory T cells at day 30 were positively correlated with acute GVHD. PBMCs from allo-HSCT recipients were stimulated with or without PMA and ionomycin, and phosphorylation of ERK1/2 was analyzed by flow cytometry (gating on CD4$^+$, CD8$^+$, and CD19$^+$ cells). Closed circles, patients with GVHD; open circles, patients without GVHD. (A) Representative histograms showing CD4$^+$ T cells from patients with and without GVHD are presented. Aggregated percentages of cells from respective cell subsets expressing phospho-ERK1/2 are shown. Samples stimulated with PMA and ionomycin are shown in gray, and unstimulated samples are shown in red. Percentages of CD4$^+$ T cells (B), CD8$^+$ T cells (C), CD19$^+$ B cells (D), CD4$^+$-naive T cells (E), CD4$^+$ central memory T cells (F), and CD4$^+$ effector memory T cells (G) (n = 10 per group; data are expressed as means ± SEMs). (H) Clinical course of GVHD, immunosuppressants used and phospho-ERK1/2 levels in CD4$^+$ T cells at days 30 and 60. *$P < .05$; **$P < .01$ (unpaired 2-tailed Student *t* test). n.s., not significant.

Several studies indicate that alloreactivity of premature T cells without any antigenic memory might trigger GVHD after allo-HSCT.[10-12] In addition, central memory T cells may play a role in GVHD.[13] We previously showed that MEK inhibitors suppress GVHD without abrogating antiviral/antitumor immunity by selectively suppressing naïve/central memory T cells.[6,7] Here, our results point specifically to the clinical significance of the MEK/ERK pathway within these cell subsets. Although several biomarkers have been raised as for acute GVHD,[14-16] none has been validated as a predictor of either the risk of developing acute GVHD or responsiveness to treatment. We suggest that the risk for GVHD and the therapeutic effects of drugs can be evaluated by analyzing the activation status of the RAS/MEK/ERK signaling pathway in CD4$^+$ T cells; that is, this may be a marker of the development and persistence of acute GVHD.

We examined the association between the RAS/MEK/ERK pathway activation in T and B lymphocytes and occurrence of acute GVHD after allo-HSCT. Notably, phosphorylation of ERK1/2 in CD4$^+$ T cells was positively associated with acute GVHD. In particular, phospho-ERK1/2 levels in naive and central memory CD4$^+$ T cells were associated strongly with acute GVHD. Although further validation is still needed, these results suggest that the RAS/MEK/ERK signaling plays a significant role in human GVHD and provide a possible rationale for the efficacy of MEK inhibitors against GVHD.

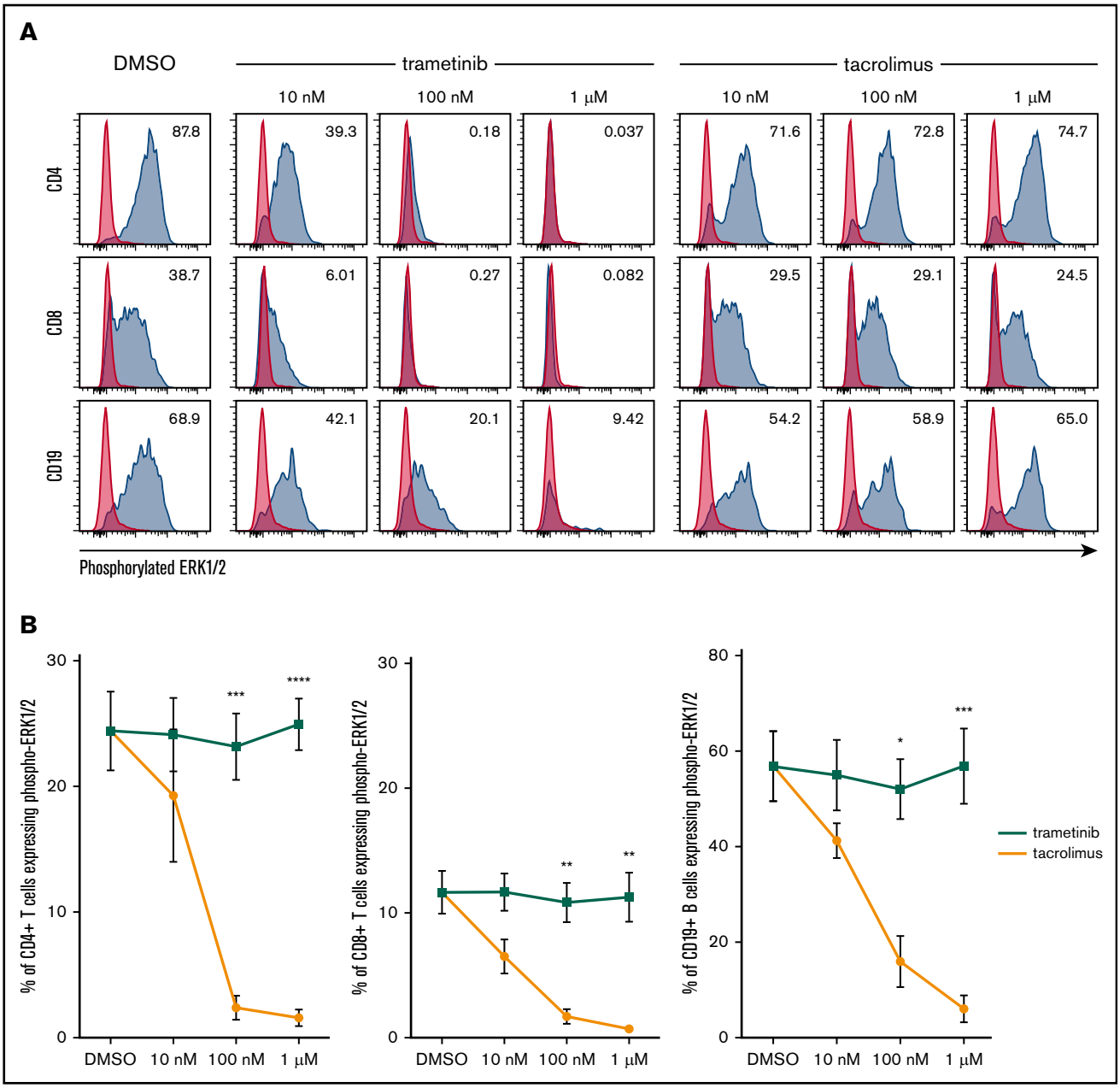

Figure 2. Trametinib suppresses phosphorylation of ERK1/2 in T and B cells. (A) PBMCs were isolated from patients and stimulated with PMA and ionomycin for 15 minutes. Expression of phosphorylated ERK1/2 by $CD4^+$ T, $CD8^+$ T cells, and $CD19^+$ B cells was analyzed after treatment (or not) with trametinib and tacrolimus (10 nM, 100 nM, and 1 μM). The numbers represent the frequencies of phospho-ERK1/2 cells compared with controls. (B) Aggregated percentages of cells $CD4^+$/$CD8^+$ T and $CD19^+$ B cells showing phosphorylated ERK1/2 expression with PMA/Ionomycin stimulation for 4 hours (data expressed as means ± SEMs; n = 4 per group, gray; PMA and ionomycin stimulation, red; unstimulated). *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$ (unpaired 2-tailed Student *t* test).

Acknowledgments

The authors thank all the patients and investigators at participating institutions.

This work was supported by research grants from MEXT KAKENHI (26461451) (T.S.), Takeda Science Foundation, SENSHIN Medical Research Foundation, Kobayashi Cancer Research Foundation, Bristol-Myers Squibb Foundation (T.S.), and Kanae Foundation for the Promotion of Medical Science (H.I.).

Authorship

Contribution: H.I. saw the patients, collected and analyzed the data, performed experiments, and wrote the manuscript; T.S. conceived the primary idea, designed the research, saw the patients, analyzed the data, and wrote the manuscript; S.Y. and T.I. saw the patients, collected the samples, and contributed to manuscript editing; and S.K. supervised the research and contributed to manuscript editing.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profile: T.S., 0000-0002-2085-6151.

Correspondence: Takero Shindo, Department of Hematology/Oncology, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan; e-mail: takeros@kuhp.kyoto-u.ac.jp.

References

1. Appelbaum FR. Haematopoietic cell transplantation as immunotherapy. *Nature*. 2001;411(6835):385-389.
2. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol*. 2007;7(5):340-352.
3. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. 2012;12(6):443-458.
4. Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. Cytomegalovirus in hematopoietic stem cell transplant recipients: Current status, known challenges, and future strategies. *Biol Blood Marrow Transplant*. 2003;9(9):543-558.
5. Inamoto Y, Flowers ME, Lee SJ, et al. Influence of immunosuppressive treatment on risk of recurrent malignancy after allogeneic hematopoietic cell transplantation. *Blood*. 2011;118(2):456-463.
6. Shindo T, Kim TK, Benjamin CL, Wieder ED, Levy RB, Komanduri KV. MEK inhibitors selectively suppress alloreactivity and graft-versus-host disease in a memory stage-dependent manner. *Blood*. 2013;121(23):4617-4626.
7. Itamura H, Shindo T, Tawara I, et al. The MEK inhibitor trametinib separates murine graft-versus-host disease from graft-versus-tumor effects. *JCI Insight*. 2016;1(10):e86331.
8. Takahagi A, Shindo T, Chen-Yoshikawa TF, et al. Trametinib attenuates delayed rejection and preserves thymic function in rat lung transplantation. *Am J Respir Cell Mol Biol*. 2019;61(3):355-366.
9. Infante JR, Fecher LA, Falchook GS, et al. Safety, pharmacokinetic, pharmacodynamic, and efficacy data for the oral MEK inhibitor trametinib: a phase 1 dose-escalation trial. *Lancet Oncol*. 2012;13(8):773-781.
10. Anderson BE, McNiff J, Yan J, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest*. 2003;112(1):101-108.
11. Bleakley M, Heimfeld S, Jones LA, et al. Engineering human peripheral blood stem cell grafts that are depleted of naïve T cells and retain functional pathogen-specific memory T cells. *Biol Blood Marrow Transplant*. 2014;20(5):705-716.
12. Dutt S, Tseng D, Ermann J, et al. Naive and memory T cells induce different types of graft-versus-host disease. *J Immunol*. 2007;179(10):6547-6554.
13. Shindo T, Ishikawa T, Fukunaga A, Hori T, Uchiyama T. Growth and differentiation advantages of CD4+ OX40+ T cells from allogeneic hematopoietic stem cell transplantation recipients. *Biol Blood Marrow Transplant*. 2008;14(3):268-281.
14. Levine JE, Paczesny S, Sarantopoulos S. Clinical applications for biomarkers of acute and chronic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2012;18(1Suppl):S116-S124.
15. Chen Y-B, Cutler CS. Biomarkers for acute GVHD: can we predict the unpredictable? *Bone Marrow Transplant*. 2013;48(6):755-760.
16. Paczesny S, Raiker N, Brooks S, Mumaw C. Graft-versus-host disease biomarkers: omics and personalized medicine. *Int J Hematol*. 2013;98(3):275-292.