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Research Article

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The efficacy of allogeneic hematopoietic stem cell transplantation for hematologic malignancies is limited by the difficulty in suppressing graft-versus-host disease (GVHD) without compromising graft-versus-tumor (GVT) effects. We previously showed that RAS/MEK/ERK signaling depends on memory differentiation in human T cells, which confers susceptibility to selective inhibition of naive T cells. Actually, antineoplastic MEK inhibitors selectively suppress alloreactive T cells, sparing virus-specific T cells in vitro. Here, we show that trametinib, a MEK inhibitor clinically approved for melanoma, suppresses GVHD safely without affecting GVT effects in vivo. Trametinib prolonged survival of GVHD mice and attenuated GVHD symptoms and pathology in the gut and skin. It inhibited ERK1/2 phosphorylation and expansion of donor T cells, sparing Tregs and B cells. Although high-dose trametinib inhibited myeloid cell engraftment, low-dose trametinib suppressed GVHD without severe adverse events. Notably, trametinib facilitated the survival of mice transplanted with allogeneic T cells and P815 tumor cells with no residual P815 cells observed in the livers and spleens, whereas tacrolimus resulted in P815 expansion. These results confirm that trametinib selectively suppresses GVHD-inducing T cells while sparing antitumor T cells in vivo, which makes it a promising candidate for translational studies aimed at preventing or treating GVHD.

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The MEK inhibitor trametinib separates murine graft-versus-host disease from graft-versus-tumor effects

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The efficacy of allogeneic hematopoietic stem cell transplantation for hematologic malignancies is limited by the difficulty in suppressing graft-versus-host disease (GVHD) without compromising graft-versus-tumor (GVT) effects. We previously showed that RAS/MEK/ERK signaling depends on memory differentiation in human T cells, which confers susceptibility to selective inhibition of naive T cells. Actually, antineoplastic MEK inhibitors selectively suppress alloreactive T cells, sparing virus-specific T cells in vitro. Here, we show that trametinib, a MEK inhibitor clinically approved for melanoma, suppresses GVHD safely without affecting GVT effects in vivo. Trametinib prolonged survival of GVHD mice and attenuated GVHD symptoms and pathology in the gut and skin. It inhibited ERK1/2 phosphorylation and expansion of donor T cells, sparing Tregs and B cells. Although high-dose trametinib inhibited myeloid cell engraftment, low-dose trametinib suppressed GVHD without severe adverse events. Notably, trametinib facilitated the survival of mice transplanted with allogeneic T cells and P815 tumor cells with no residual P815 cells observed in the livers and spleens, whereas tacrolimus resulted in P815 expansion. These results confirm that trametinib selectively suppresses GVHD-inducing T cells while sparing antitumor T cells in vivo, which makes it a promising candidate for translational studies aimed at preventing or treating GVHD.

Introduction

Allogeneic hematopoietic stem cell transplantation is a curative treatment capable of inducing long-term remission in patients with therapy-resistant hematopoietic malignancies; however, graft-versus-host disease (GVHD) limits its indication and success (1). The pathophysiology of GVHD involves several factors, such as inflammatory cytokines, chemokines, thymic function, and humoral immunity, although the primary trigger is thought to be alloreactivity of donor T cells against host antigens (2). On the other hand, donor T cells are important for maintaining antiviral immunity and graft-versus-tumor (GVT) effects against residual host tumor cells (2). Transplants with T cell-depleted BM (TCD-BM) result in increased infection, and recipients without GVHD have a higher incidence of relapses of the underlying malignancies (3, 4). Furthermore, calcineurin inhibitors and corticosteroids that are used to prevent GVHD suppress T cell immunity nonspecifically and limit protective immunity and GVT effects. Therefore, the selective inhibition of GVHD without compromising GVT effects is desirable. However, a reliable method to achieve these goals has proved elusive, in part, due to an incomplete understanding of T cell alloreactivity and immune recovery in human hematopoietic stem cell transplantation.

T cells are classified into naive T cells that have not yet encountered antigen and central/effector memory T cells with antigenic memory (5). They may be phenotypically defined according to the surface expression of CD45RA, CCR7, CD27, and CD62L in humans (6) and CD44 and CD62L in mice (7). In mice, the transfer of naive T cells results in GVHD, whereas that of memory T cells does not (8–10), but still preserves GVT effects (11). In humans, selective depletion of naive T cells suppresses alloreactivity in vitro (12, 13), while memory T cells are involved in antimicrobial immunity (14). Thus, selective suppression of

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naive T cells while sparing memory T cells may enable the selective inhibition of GVHD without affecting GVT effects in human hematopoietic stem cell transplantation.

As the RAS/MEK/ERK pathway is activated in many types of cancers, many MEK inhibitors that target this pathway have been developed (15). In a mouse model of GVHD, this pathway was activated in T cells (16). We previously investigated the phosphorylation status of ERK1/2 during the activation of human T cells and found that the RAS/MEK/ERK pathway is preferentially activated in naive and central memory T cells but not in effector memory T cells (17). We then confirmed that MEK inhibitors selectively suppressed alloreactivity of T cells while sparing herpesvirus-specific T cells and showed that the MEK inhibitor selumetinib delayed the onset of murine GVHD (17).

While GVHD-induced lethality was delayed, selumetinib did not prevent the occurrence of GVHD; whether long-term administration of MEK inhibitors is feasible and ameliorates GVHD manifestations is still unclear. Furthermore, how MEK inhibition modulates immunity *in vivo* and whether it spares GVT effects remain to be elucidated. In this report, we show that a new MEK inhibitor trametinib (GSK1120212) selectively suppressed GVHD without abrogating GVT effects in several murine BM transplant (BMT) models. We also show that long-term administration of low-dose trametinib was safe and did not affect BM engraftment. Trametinib prolonged the survival of relapsing malignant melanoma patients (18) and gained FDA approval for clinical use in 2013. To date, few adverse effects have been reported except for mild diarrhea and skin rashes (18–21). Current data support that MEK inhibition by trametinib may be a promising strategy for translational studies aimed at preventing or treating GVHD while sparing GVT effects in humans.

Results

Trametinib suppresses gut GVHD after haploidentical murine transplantation. The effects of trametinib were first examined in an MHC-haploidentical GVHD model. (C57BL/6 × DBA/2) F1 hybrid (B6D2F1; H-2^{b/d}) mice were transfused with C57BL/6 (B6; H-2^{b/b}) BM and T cells after total body irradiation (TBI) (10.5 Gy). Control BMT mice received BM alone after TBI. Vehicle-treated mice in the GVHD group developed gut GVHD symptoms due to MHC mismatch within 1 month, following a clinical syndrome characterized by continuous diarrhea and severe body weight loss; these mice died after 2 months (Figure 1, A and B: BM+T, vehicle).

Vehicle or trametinib was administered by oral gavage in 2 doses of 0.1 and 0.3 mg/kg/d from day 0 through day 30. Both trametinib dosing groups showed significantly prolonged survival (Figure 1A, $P < 0.01$ [0.1 mg/kg] and $P < 0.001$ [0.3 mg/kg]), decreased body weight loss (Figure 1B, $P < 0.01$ and $P < 0.001$), and decreased clinical GVHD score (Figure 1C, $P < 0.01$ and $P < 0.001$).

Flow cytometric analyses of peripheral blood on day 10 revealed highly phosphorylated ERK1/2 in CD4⁺ and CD8⁺ T cells in vehicle-treated mice, which was suppressed in trametinib-treated mice in a dose-dependent manner (Figure 1D). Flow cytometric analyses of T cell subsets in the spleen showed that, in the GVHD mice (BM+T, vehicle), CD8⁺ T cells expanded and dominated over CD4⁺ T cells, whereas trametinib treatment at 0.3 mg/kg/d suppressed the expansion of CD8⁺ T cells (Figure 1E). Notably, while most CD4⁺ and CD8⁺ T cells had an effector memory T cell phenotype (CD62L[−] cells) and naive T cells (CD62L⁺ cells) were reduced in the GVHD mice, substantial populations of naive T cells were still detected in the trametinib-treated mice.

On day 35, H&E staining of the colonic mucosa in the vehicle group demonstrated alterations in the mucous membrane, lymphocyte infiltration in the submucosal layer, loss of polarity, and apoptotic bodies, whereas trametinib attenuated these effects (Figure 1, F and G). These findings show that trametinib suppressed gut GVHD by inhibiting the activation of the RAS/MEK/ERK pathway and suggest that it suppressed the functional differentiation of naive T cells into memory T cells *in vivo*.

Trametinib suppresses cutaneous GVHD. BALB/c mice transplanted with B10.D2 BM and T cells are mismatched at minor antigens from donors, leading recipients to develop cutaneous GVHD, characterized by skin sclerosis and alopecia. Though the effect of trametinib from day 0 through day 35 was marginal in terms of survival (Figure 2A), it limited body weight loss (Figure 2B) and reduced the clinical GVHD score (Figure 2C, $P < 0.05$). Vehicle-treated mice developed sclerotic skin, alopecia, and cutaneous erosion around the anus, which was suppressed by trametinib (Figure 2D). H&E staining of skin samples showed that trametinib suppressed the thickening of collagen layers, the disturbance of the subcutaneous architecture, and small lymphocyte infiltration in the dermis (Figure 2E). Flow cytometric analyses of peripheral blood on day 21

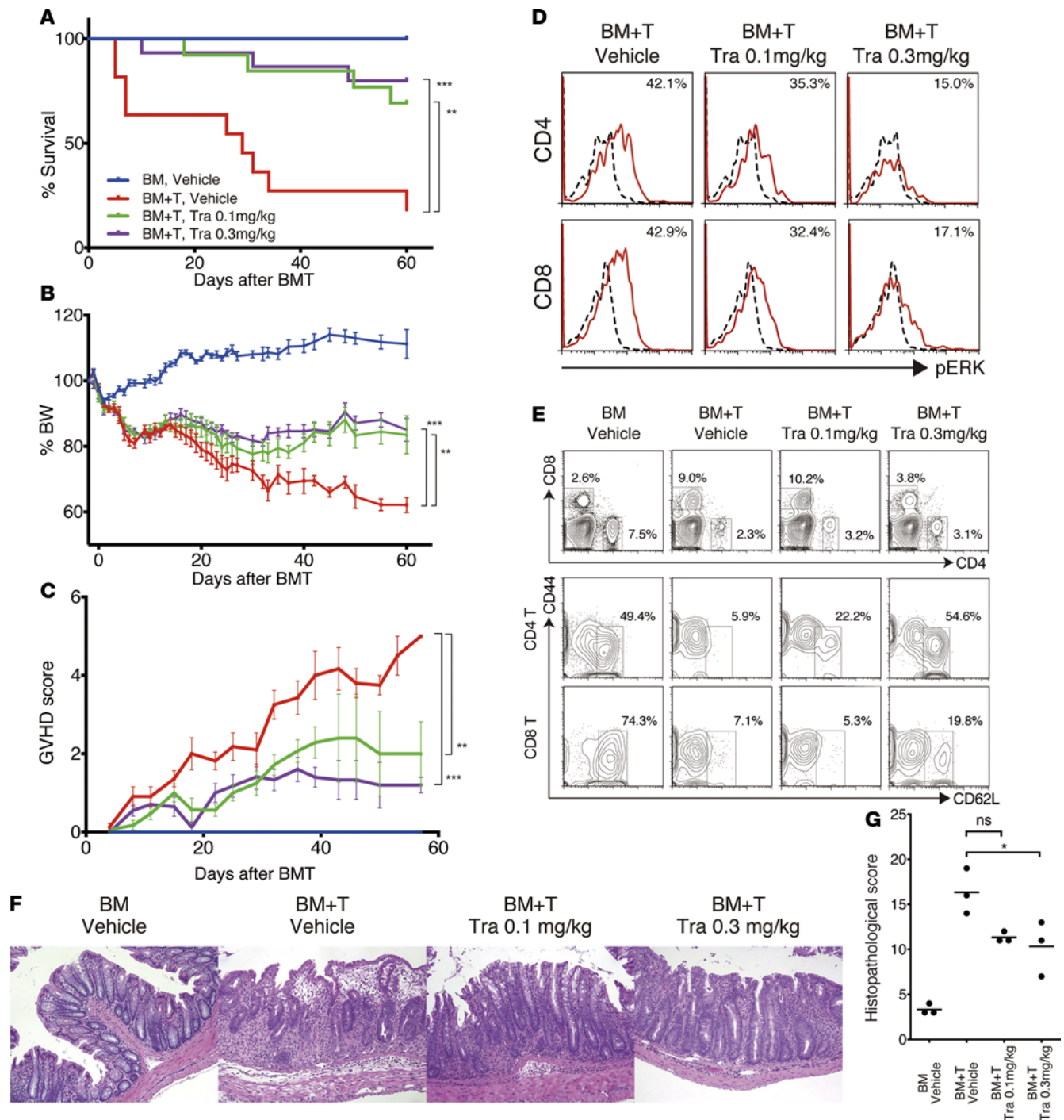


Figure 1. Trametinib suppressed gut graft-versus-host disease and spared naive T cells. B6D2F1 recipients were irradiated (10.5 Gy) on day -1 and infused (on day 0) with 5×10^6 B6 BM cells and 2×10^6 B6 T cells. Recipients were administered vehicle or trametinib (0.1 or 0.3 mg/kg) once daily via oral gavage from day 0 through day 30. Combined data of 2 independent experiments ($n = 11$ in total) are shown for survival (with the log-rank test) (**A**), percentage body weight (BW) (with the 2-tailed Student's *t* test at day 60) (**B**), and clinical graft-versus-host disease (GVHD) score (with the 2-tailed Student's *t* test at day 58) (**C**). (**D**) Phosphorylation of ERK1/2 within CD4⁺ and CD8⁺ T cells in the recipient peripheral blood was assessed by flow cytometry on day 10. Dashed lines represent the results of the recipients of BM cells only, and percentages represent the frequencies of positive cells calculated by the Overton cumulative histogram subtracting algorithm. Data are representative of 3 independent experiments. (**E**) CD4/CD8 and naive/memory T cell subsets of splenocytes from the recipients on day 28 were analyzed by flow cytometry. The percentages represent the frequencies of CD4⁺, CD8⁺, naive (CD62L⁺CD44⁻), and memory (CD62L⁻CD44⁺) cells, respectively. Data are representative of 2 independent analyses. (**F**) H&E staining of the colonic mucosa of each group. Representative images from 2 independent experiments are shown. Original magnification, $\times 100$. (**G**) Individual histopathological scores obtained from 3 mice are shown along with the means (2-tailed Student's *t* test with Bonferroni correction). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

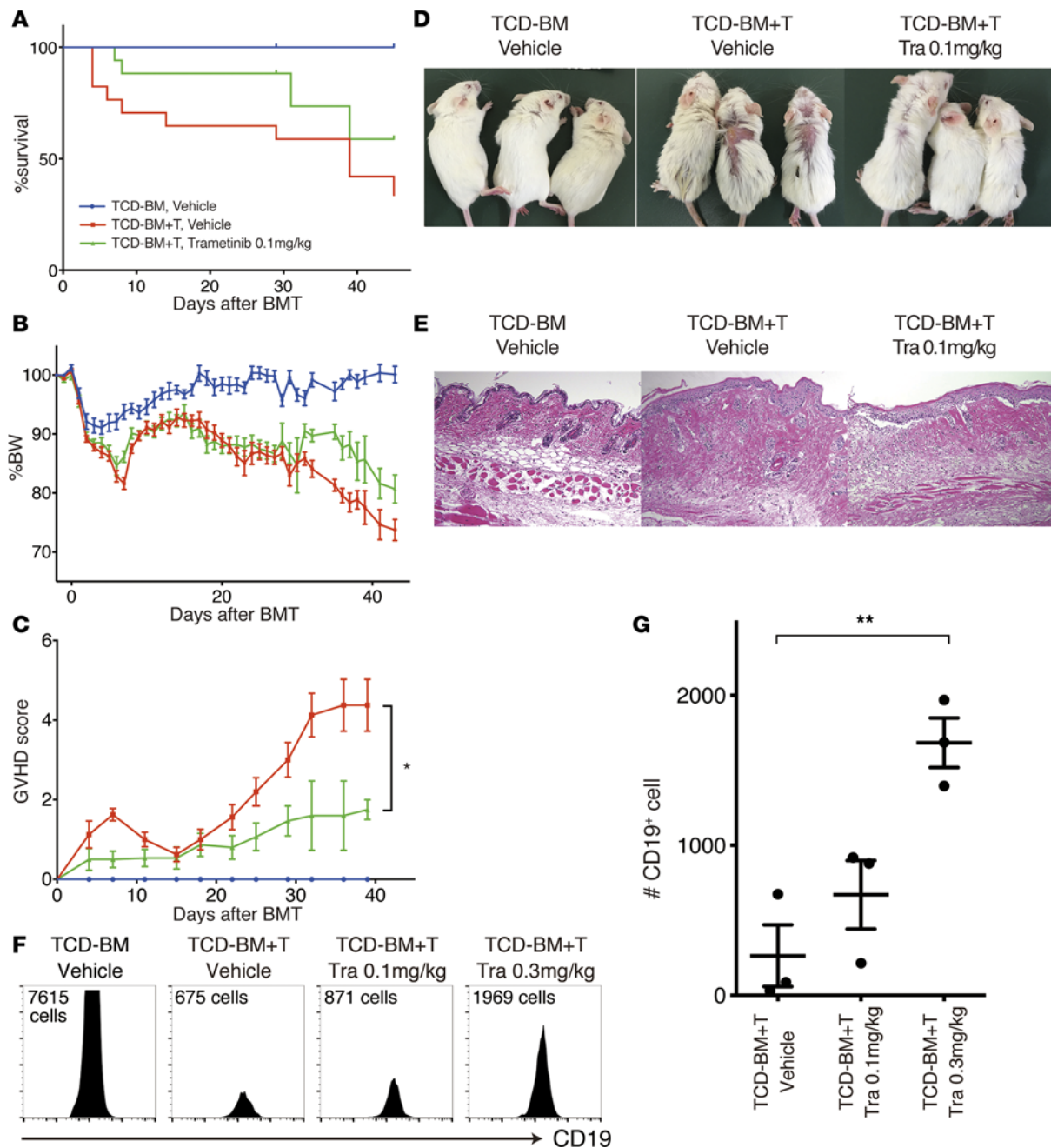


Figure 2. Trametinib suppressed cutaneous graft-versus-host disease. BALB/c recipients were irradiated (7.5 Gy) on day -1 and infused (on day 0) with 5×10^6 B10.D2 T cell-depleted BM cells (TCD-BM) and 1×10^6 T cells (BM only, vehicle: $n = 11$, trametinib, 0.1 mg/kg: $n = 15$). Recipients were administered vehicle or trametinib (0.1 or 0.3 mg/kg) once daily via oral gavage from day 0 through 30. Aggregated data of 2 independent experiments are shown for percentage survival (log-rank test) (A), percentage body weight (BW) (2-tailed Student's t test at day 41) (B), and clinical graft-versus-host disease (GVHD) score (2-tailed Student's t test at day 38) (C). Representative images of fur texture and alopecia (D) and H&E staining of the skin (E) of the recipients on day 70 are shown. Original magnification, $\times 100$. The percentage and number of CD19⁺ B cells gating on 1.5×10^4 lymphocytes in the BM cell population on day 21 are shown as representative histograms along with the cell numbers (F) and individual plots \pm SEM (G) from 3 independent experiments (2-tailed Student's t test with Bonferroni correction) (G). * $P < 0.05$, ** $P < 0.01$.

showed that the number of CD19⁺ B cells was reduced due to inflammation in the BM of GVHD mice. However, substantial numbers of B cells were detected in a trametinib dose-dependent manner (Figure 2, F and G, $P < 0.05$), suggesting that trametinib suppressed inflammation in BM and facilitated B cell engraftment.

High-dose, but not low-dose, trametinib suppresses myeloid cell engraftment. In nude mice, daily administration of 10–30 mg/kg trametinib is well tolerated, with only mild body weight loss (22). However, inhibition of the RAS/MEK/ERK pathway was suggested to suppress erythropoiesis (23). Therefore, the possibility that

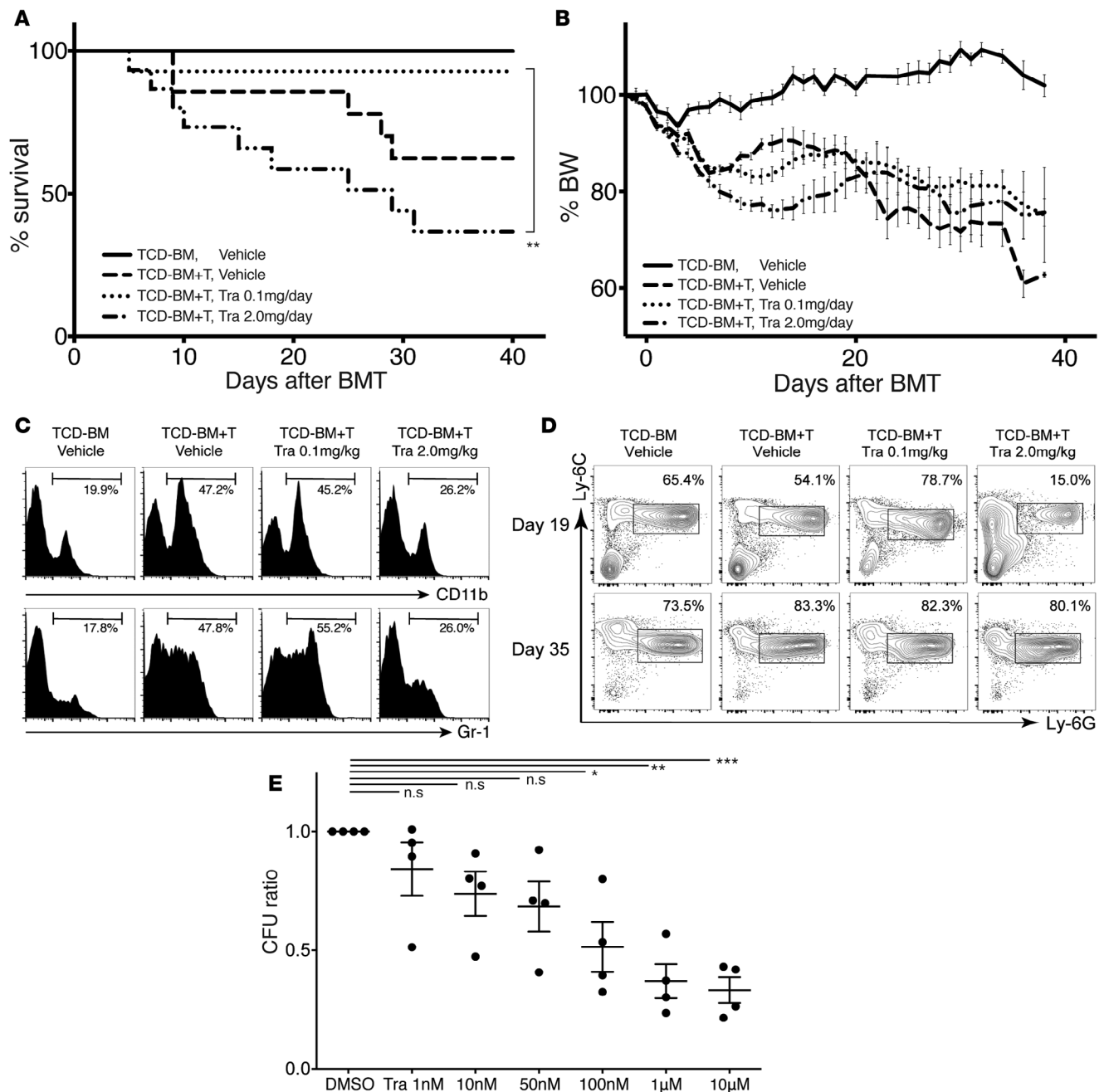


Figure 3. High-dose trametinib delayed myeloid cell engraftment, while long-term administration of low-dose trametinib was well tolerated. Irradiated B6D2F1 recipients were infused with C57BL/6 TCD-BM with or without T cells, and vehicle or trametinib at 0.1 or 2.0 mg/kg was administered from day 0 through day 30. Combined data of 2 independent experiments ($n = 12$ in each group) are shown for survival (log-rank test) (A) and percentage body weight (BW) (2-tailed Student's t test at day 37) (B). (C) The percentages of CD11b⁺ and Gr-1⁺ cells among BM cells were assessed on day 14 by flow cytometry. Data are representative of 3 independent experiments. (D) The percentages of Ly-6C⁺Ly-6G⁺ granulocytic myeloid-derived suppressor cells among CD11b⁺ cells on day 19 and day 35. (E) The numbers of colonies formed with trametinib at different concentrations were counted. Ratios were obtained by dividing the number of colonies formed with trametinib by the number of colonies formed with DMSO, and 4 individual plots are shown (mean \pm SEM) (2-tailed Student's t test with Bonferroni correction). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

trametinib may suppress the engraftment of donor hematopoietic stem cells is an issue of concern. In the present study, trametinib was administered to the B6D2F1 model at low (0.1 mg/kg) and high (2.0 mg/kg) doses. Mice treated with high-dose trametinib had severe body weight loss early after transplantation, and many of them succumbed (Figure 3, A and B, $P < 0.01$). Though diarrhea and gastrointestinal discomfort are potential adverse effects of trametinib therapy (19, 20), none of the mice experienced severe diarrhea.

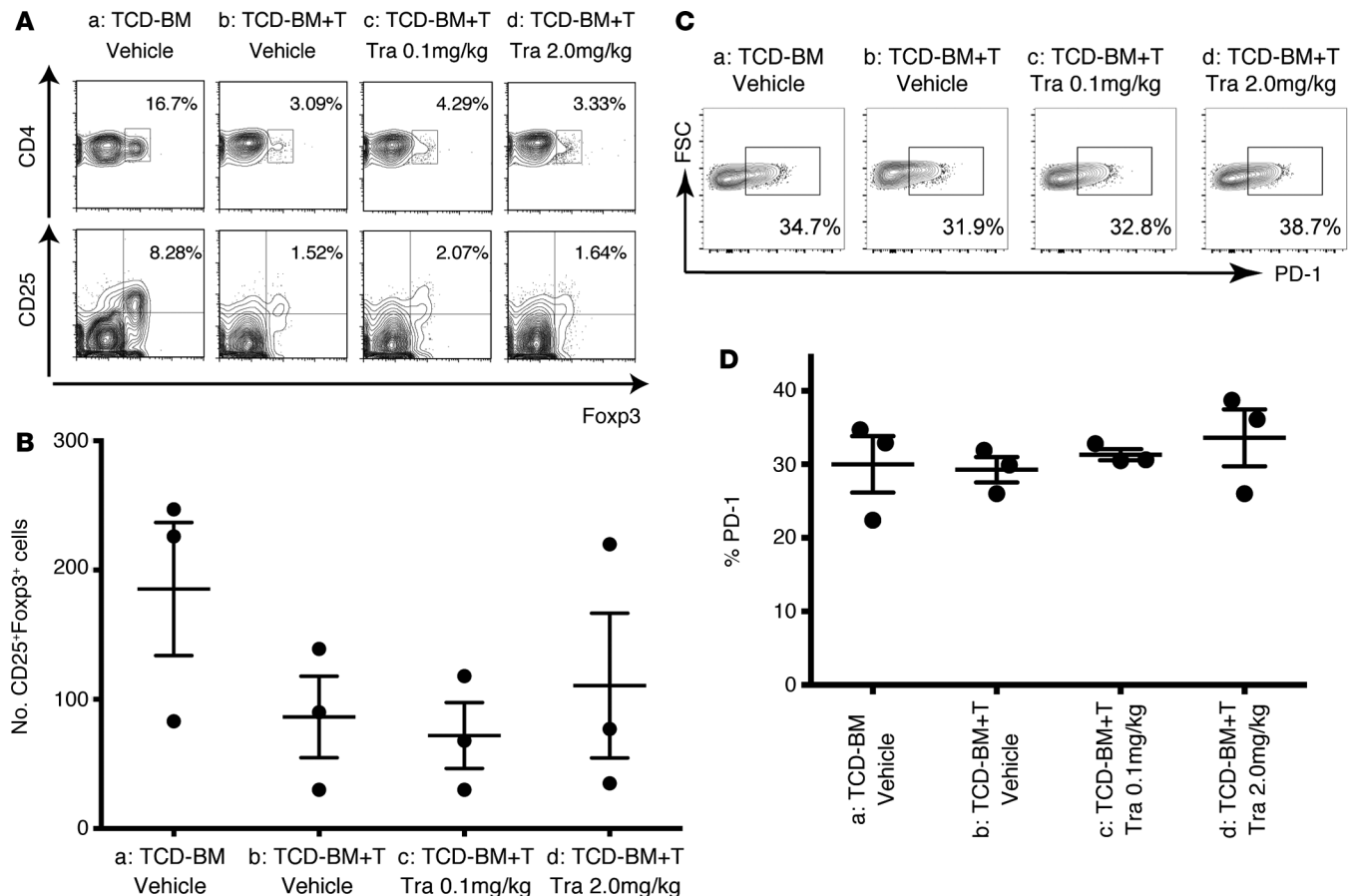


Figure 4. Trametinib did not enhance deletion of CD4⁺CD25⁺Foxp3⁺ Tregs. Irradiated B6D2F1 recipients were infused with C57BL/6 TCD-BM with or without T cells. Flow cytometric analyses detected CD4⁺CD25⁺Foxp3⁺ Tregs and the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs gating on CD4⁺ T cells in the spleens in a representative analysis (**A**), and the aggregated absolute numbers among 1×10^4 lymphocytes are shown (mean ± SEM) (**B**) in 3 independent experiments. (**C** and **D**) Irradiated B6D2F1 recipients were infused with C57BL/6 TCD-BM with or without T cells, and the expression of PD-1 on CD4⁺CD25^{hi} Tregs was analyzed by flow cytometry. Representative data from 3 analyses are shown (**C**), and the percentages of PD-1-positive cells are plotted (mean ± SEM) (**D**). Statistical analyses were performed with the 2-tailed Student's *t* test with Bonferroni correction in **B** and **D**, and no significant differences were detected.

Flow cytometric analyses showed that the number of Gr-1- and CD11b-positive myeloid cells decreased in high-dose trametinib-treated mice (Figure 3C). Trametinib did not alter the phenotypes and frequencies of hematopoietic stem cell subsets by flow cytometry (data not shown), indicating that MEK inhibition did not specifically eliminate stem cells, but rather delayed their engraftment. Furthermore, we analyzed subpopulations of myeloid-derived suppressor cells (MDSCs) that were positive for Gr-1 and CD11b. These cells inhibit alloreactions and expand to achieve hematopoietic chimerism after BMT (24, 25); this suggests that they contribute to smooth engraftment. In particular, the population of granulocytic MDSCs with a CD11b⁺Ly6G⁺Ly6C^{lo} phenotype was transiently reduced by high-dose (2.0 mg/kg) trametinib, while low-dose (0.1 mg/kg) trametinib spared the CD11b⁺Ly6G⁺Ly6C^{lo} population (Figure 3D). Finally, *in vitro* colony assays showed that colony formation was suppressed by high-dose trametinib (Figure 3E, $P < 0.01$ and $P < 0.001$). These findings suggest that high-dose trametinib might delay engraftment of donor BM cells by suppressing granulocytic MDSCs, whereas low-dose trametinib is well tolerated.

Trametinib does not increase depletion of Foxp3⁺ Tregs. The importance of Tregs in transplant immunology has been increasingly recognized, and depletion of Tregs is thought to result in severe GVHD and fatal complications. Therefore, whether MEK inhibition suppresses Tregs is an important question. To this end, we performed flow cytometric analyses with splenocytes of B6→B6D2F1 model mice. As shown in Figure 4, A and B, compared with the non-GVHD mice transplanted with TCD-BM only (group a), the GVHD mice transplanted with TCD-BM and T cells (groups b–d) showed decreased CD4⁺CD25⁺Foxp3⁺ Tregs when assessed by both frequencies and absolute numbers. However, among the GVHD mice,

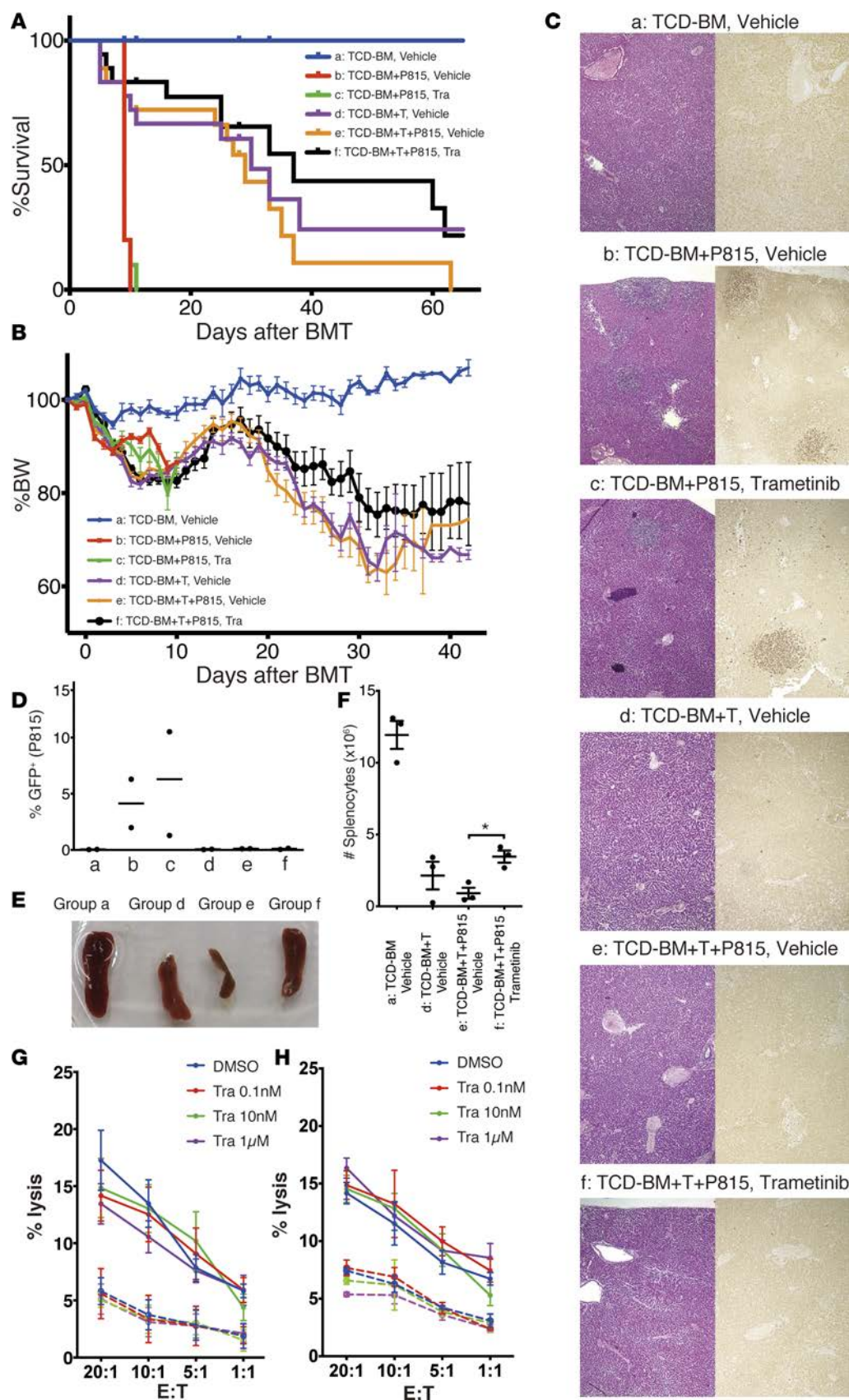


Figure 5. Trametinib spared graft-versus-tumor effects. Irradiated B6D2F1 recipients were infused with C57BL/6 TCD-BM with or without T cells, with or without P815 tumor cells. Vehicle or trametinib at 0.1 mg/kg was administered from day 0 through day 30. Combined data of 2 independent experiments (group a: $n = 10$, groups b–f: $n = 18$) are shown for survival (A) and percentage body weight (BW) (B). Statistical analyses were performed with the log-rank test (A) and the 2-tailed Student's t test at day 42 (B), and no significant differences were detected. (C) H&E (left) and GFP (right) staining of the livers on day 9. Representative images of 3 analyses are shown. Original magnification, $\times 100$. (D) P815 cells in the spleens on day 10 were detected as GFP⁺ cells by flow cytometry. Data are expressed as the percentages from 2 independent experiments, along with the mean value. (E and F) The spleens were harvested from 3 mice per group on day 33 (E), and cell numbers are expressed as the mean \pm SEM (2-tailed Student's t test with Bonferroni correction) (F). (G and H) T cells (G) or NK cells (H) from C57BL/6 (allogeneic; solid lines: $n = 6$) or DBA/2 (syngeneic; dashed lines: $n = 3$) mice were cocultured with P815 cells at different ratios. DMSO or trametinib at various concentrations was added to the media, and the percentage lysis of P815 cells was calculated by detecting PI-positive cells among CFSE-labeled cells. * $P < 0.05$.

trametinib-treated mice (groups c and d) still contained some Tregs compared with vehicle-treated mice (group b). Thus, trametinib did not enhance depletion of Tregs.

Besides cell numbers, we also investigated whether trametinib modulates exhaustion markers (i.e., PD-1, Tim-3, and Lag-3) expressed by CD4⁺CD25^{hi} Tregs. Trametinib did not modulate PD-1 in vivo (Figure 4, C and D), although it downregulated expression of Tim-3 and Lag-3

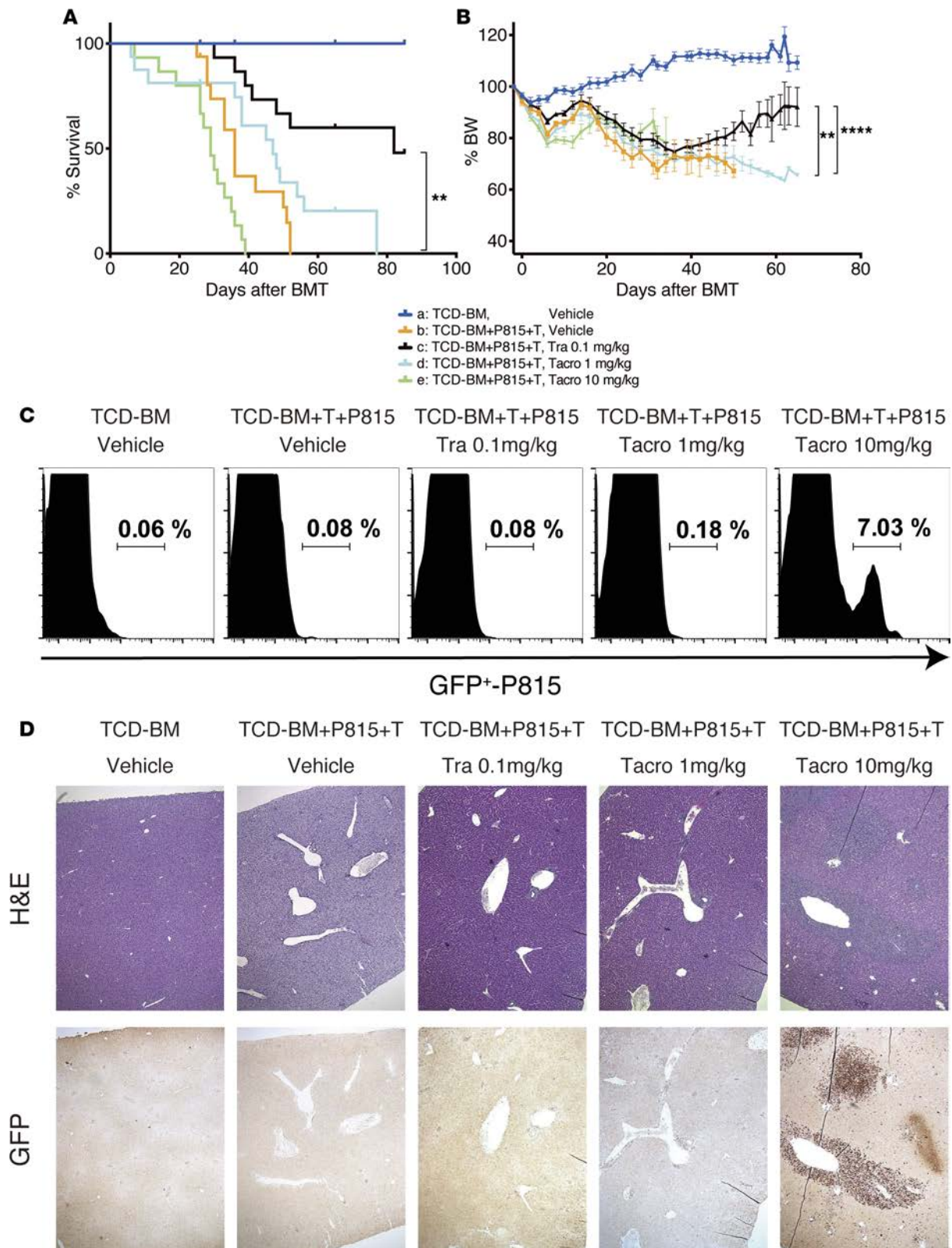


Figure 6. Trametinib suppressed graft-versus-host disease sparing graft-versus-tumor effects, whereas tacrolimus abrogated graft-versus-tumor effects. B6D2F1 recipients were infused with C57BL/6 TCD-BM with or without T cells with P815 tumor cells after irradiation, and vehicle, tacrolimus at 1.0 or 10 mg/kg, or trametinib at 0.1 mg/kg was administered from day 0 through day 50. Combined data of 2 independent experiments (group a: $n = 8$, groups b–e: $n = 15$) are shown as for survival (log-rank test) (A) and percentage body weight (BW) (2-tailed Student's t test at day 65) (B). (C) Flow cytometric analysis was performed to detect GFP⁺-P815 cells in the spleen on day 37. (D) H&E and GFP staining of the livers on day 37 is shown. (C and D) Data are representative of 2 independent analyses. ** $P < 0.01$, **** $P < 0.0001$.

compared with that in vehicle-treated GVHD mice; however, the differences were not significant (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/jci.insight.86331DS1). Although these data should be verified and their meaning should be clarified, it is possible that trametinib not only spares some Tregs, but also avoids exhausting Tregs.

Trametinib spares GVT effects. Inoculation of P815 cells, a mastocytoma cell line, with B6 TCD-BM and T cells to B6D2F1 mice enables the comparison of the GVT effects of B6 T cells against P815 cells. As shown in Figure 5, A and B, mice transplanted with TCD-BM and P815 cells died early (i.e., by day 15; group b), whereas simultaneous transplantation of B6 T cells prolonged survival (group e). Compared with group d (transplanted with TCD-BM and T cells, vehicle-treated), simultaneous transfusion of P815 cells resulted in impaired survival (group e), which may be due to worsening of GVHD due to cytokine storm associated with tumor cell infusion (26). Notably, trametinib treatment of the recipients that received TCD-BM and P815 and B6 T cells (group f) showed similar or even longer survival compared with that of the vehicle-treated recipients (group e). On the other hand, after trametinib treatment, the mice that received P815 cells without B6 T cells succumbed early due to tumor expansion (group c), implying that trametinib had no direct antitumor effects at a dose of 0.1 mg/kg/d, whereas it spared GVT effects. As shown in Figure 5C, H&E and GFP staining of the liver on day 9 revealed P815 cell infiltration and nodule formation in mice receiving P815 cells (groups b and c), but not in those receiving P815 and T cells (groups e and f), independently of trametinib treatment. Flow cytometric analyses of the spleen on day 10 detected substantial amounts of P815 cells in the recipients of P815 cells without T cells (Figure 5D). On the other hand, P815 cells were cleared in the recipients of P815 and T cells, regardless of the administration of vehicle or trametinib, indicating that trametinib did not weaken GVT effects. Furthermore, assessment of the spleens harvested on day 33 showed the highest exhaustion of splenocytes in group e, which received T cells and P815 cells, implying GVHD deterioration associated with tumor transfusion (26, 27). Trametinib treatment reversed this exhaustion, suggesting that it suppressed inflammation resulting from tumor injection and GVHD (Figure 5, E and F, $P < 0.05$).

Our findings indicate that low-dose trametinib (0.1 mg/kg) selectively suppresses GVHD while sparing GVT effects. This notion was supported by cytotoxicity assays, in which trametinib did not decrease cytotoxicity against P815 cells by T cells (Figure 5G) and NK cells (Figure 5H).

Trametinib separates GVT effects from GVHD, whereas tacrolimus abrogates GVT effects. Finally, we compared trametinib with tacrolimus, the calcineurin inhibitor commonly employed for GVHD prophylaxis in humans, assessing both GVHD and GVT effects. B6 TCD-BM was transplanted with B6 T cells and P815 cells to B6D2F1 mice; in addition, vehicle, trametinib (0.1 mg/kg), or tacrolimus (1.0 or 10 mg/kg) was administered daily from day 0 through day 50. Similarly as in Figure 5, A and B, the mice transplanted with TCD-BM, T cells, and P815 cells (group b) experienced severe diarrhea and body weight loss by day 40 and succumbed (Figure 6, A and B) but were not found to have P815 tumor cell expansion in the spleen (Figure 6C) or the liver (Figure 6D). Thus, B6 T cells exerted GVT effects, though the mice developed GVHD. Treatment with 1.0 mg/kg tacrolimus marginally prolonged survival, but these mice succumbed by day 80 with diarrhea (group d). Mice treated with 10 mg/kg tacrolimus had shortened survival, and substantial amounts of GFP⁺-P815 cells infiltrated the spleen (Figure 6C) and perivascular area in the liver (Figure 6D). Interestingly, some of those mice experienced paraplegia, the likely result of invasion of P815 cells into the CNS (data not shown). On the other hand, 0.1 mg/kg trametinib-treated mice demonstrated significantly prolonged survival without expansion of P815 cells in peripheral blood and the liver ($P < 0.01$, $P < 0.0001$). Though some mice succumbed, surviving mice still gained body weight by day 60 (Figure 6B), a result of resolution of GVHD. Collectively, these results demonstrate that optimal dosing of tacrolimus impairs GVT effects, while low-dose trametinib spares GVT effects but suppresses GVHD better than sub-optimal tacrolimus dosing.

Discussion

Immunosuppression is critical to safely achieving donor cell engraftment after hematopoietic stem cell transplantation; however, separation of GVHD and GVT effects is difficult. Based on our previous findings on the effects of U0126 and selumetinib, mainly with human cells (17), we investigated whether the MEK inhibitor trametinib selectively suppresses GVHD while sparing GVT effects in murine BMT models. Trametinib selectively suppresses ERK1/2 phosphorylation through the RAF-dependent activation of MEK1/2 (22, 28, 29). It was selected because of its low IC₅₀ (22, 28) and the fact that once daily adminis-

tration sufficiently suppresses ERK1/2 phosphorylation *in vivo* (29).

Our previous work did not examine the feasibility of long-term administration of MEK and whether such dosing could significantly suppress (rather than delay onset of) GVHD manifestations. In the present study, we demonstrate conclusively that treatment with low-dose trametinib for 30 days significantly suppresses GVHD in multiple murine BMT models (Figures 1 and 2). Trametinib inhibited the differentiation of T cells and expansion of CD8⁺ T cells (Figure 1), while sparing B cell engraftment and reconstitution of Tregs (Figures 2 and 4). High-dose trametinib disrupted myeloid cell engraftment, though this was not observed with administration of lower doses, which still suppressed GVHD (Figure 3). Finally, trametinib treatment allowed the retention of GVT effects (Figure 5), whereas tacrolimus abrogated GVT effects (Figure 6). As the inhibitory effects of trametinib are exclusively specific for MEK1/2 (28), it is unlikely that these effects derive from other off-target interactions. Our report paves a path to selectively suppress GVHD while preserving GVT effects pharmacologically.

The RAS/MEK/ERK pathway is activated in T cells in a murine model of GVHD (16), which we confirmed (Figure 1D). Our results indicate that pharmacological MEK inhibition suppresses major and minor antigen mismatched murine GVHD. And it is interesting that trametinib reduced early deaths by day 10 after BMT, though clinical GVHD scores were still minimal (Figure 1, A and C). It is possible that MEK inhibition suppresses cytokine-releasing syndrome right after BMT, which is consistent with our previous results (17) and would be beneficial in the clinic.

The RAS/MEK/ERK pathway is constitutively activated in many hematologic malignancies, and MEK inhibitors have shown antitumor potency against leukemia and lymphoma (30–32). In contrast to calcineurin inhibitors (33) or corticosteroids, MEK inhibitors may be less likely to increase the relapse of underlying malignancies after hematopoietic stem cell transplantation. Furthermore, another limitation of calcineurin inhibitors is that they reduce GVT effects, increasing the risk of relapse of underlying malignancies (Figure 6). Trametinib preserves GVT effects in the same settings, which makes it an attractive alternative (or adjunct) to currently available immunosuppressive agents.

MEK inhibitors affect human T cells in a memory stage-dependent manner, i.e., they selectively inhibit naive and central memory T cells while sparing effector memory T cells (17). In our murine GVHD models, most T cells had differentiated into effector memory T cells; however, some naive T cells were still detected in the spleens of trametinib-treated mice (Figure 1D), implying that trametinib suppressed the functional differentiation of naive T cells into effector memory T cells. These findings are consistent with our observations in human T cells *in vitro* and suggest that memory stage-dependent suppression of MEK inhibition occurs *in vivo*.

Studies have shown that MEK inhibition induces Foxp3⁺ Tregs (34, 35). These findings were not confirmed (Figure 4), which could be attributed to the complexity of the murine BMT models. However, the fact that MEK inhibition still preserves some Foxp3⁺ Tregs may be beneficial in hematopoietic stem cell transplantation, given the established importance of Tregs in limiting GVHD and support stem cell engraftment (36).

Nonspecific T cell depletion in the setting of hematopoietic stem cell transplantation increases the rate of infection and the risk of relapse of underlying hematologic malignancies (4, 37). Therefore, methods to selectively deplete CD45RA⁺ naive T cells from the graft to reduce acute and chronic GVHD are being developed (12, 13). However, the cost of *ex vivo* selective depletion is high, and it is unclear whether this method can reduce chronic GVHD because chronic GVHD is assumed to originate from T cells that differentiate from graft-derived hematopoietic stem cells. Our strategy with trametinib is unique in that GVHD could be selectively suppressed pharmacologically.

In addition to T cell activation, inflammatory factors and humoral immunity are involved in GVHD. The MAPK pathway plays important roles in the production of several inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, and MEK inhibition downregulates these cytokines (38–41). Furthermore, MEK inhibition is also involved in B cell activation (42, 43). Therefore, it is reasonable to assume that MEK inhibition suppresses pathological processes, such as fibrosis and sclerosis, that are specific to chronic GVHD.

Geest et al. analyzed genetic signatures of CD34⁺ neutrophil progenitors using microarray technology and found that activation of the MEK/ERK pathway is important for their expansion and survival (44). Sui and colleagues also reported that MEK inhibition suppresses erythropoiesis (23). These findings raise concerns regarding the potential for MEK inhibition to interfere with stem cell engraftment and hematopoiesis after BMT. In our model, high-dose trametinib interfered with myeloid engraftment and transiently

reduced the population of granulocytic MDSCs, whereas low-dose trametinib had no such effect (Figure 3). This suggests that, at low doses, MEK inhibitors do not affect engraftment and hematopoiesis after transplantation. However, close attention should be paid to dosing schedules and serum concentrations to avoid adverse events in the design of clinical trials with MEK inhibitors.

Taken together, our results indicate that MEK inhibition with trametinib is an attractive therapeutic option to prevent or reduce GVHD but still suppress the relapse of underlying hematologic malignancies. Though it is not clear whether the *in vivo* results derived from murine BMT models can be directly translated into human hematopoietic stem cell transplantation, our results are consistent with the *in vitro* results with human lymphocytes (17). As trametinib has already been approved for clinical use in the treatment of therapy-resistant melanoma and the safety of trametinib has been confirmed, we believe that our findings justify the use of trametinib to prevent and/or treat GVHD in clinical trials.

Methods

Mice. C57BL/6J (B6; H-2^{b/b}) and [C57BL/6J (NCrI) × DBA/2 (NCrI)] F1 (B6D2F1; H-2^{b/d}) mice were purchased from Charles River Laboratories Japan. DBA/2 (H-2^{d/d}), B10.D2 (H-2^{d/d}, Thy1.2), and BALB/c (H-2^{d/d}) mice were purchased from Japan SLC. Mice were 10–14 weeks old and were housed in a specific pathogen-free barrier facility.

BM transplantation. Recipient B6D2F1 mice received 10.5 Gy of lethal TBI with a GammaCell 40 unit (33 cGy/min) 1 day prior to transplant and were intravenously injected with 5×10^6 BM cells or TCD-BM cells with or without 2×10^6 T cells from B6 mice. In some experiments, recipient BALB/c mice received 7.5 Gy TBI and were injected with 5×10^6 TCD BM cells and 1×10^6 T cells from B10.D2 mice. Donor BM cells were obtained from the femurs and the tibias. When required, BM cells were depleted of T cells with CD90.2 T Cell Isolation Kit Microbeads (Miltenyi Biotec) according to the manufacturer's instructions, and T cell depletion was routinely >97%. T cells from the spleens of donors were enriched with Pan T Cell Isolation Kit II Microbeads (Miltenyi Biotec) or the EasySep T Cell Enrichment Kit (STEMCELL Technologies) and were stained for T cells (anti-CD4, clone GK1.5; anti-CD8, clone 53-6-7) in order to adjust them to the appropriate density for injection. Recipient mice harvested at day 28 or after showed >90% donor cell engraftment in the spleen and BM (data not shown).

Reagents. Trametinib (GSK1120212) and tacrolimus (FK506) were purchased from Selleck Chemicals. Reagents were reconstituted in DMSO (Sigma-Aldrich) at 10 mM for *in vitro* experiments and stored at –20°C before being added to culture media. For *in vivo* experiments, reagents were reconstituted in 200 µl vehicle (methocel/polysorbate buffer) and orally administered to the mice once a day. Tacrolimus was reconstituted in the saline and intraperitoneally injected to the mice once a day.

Assessment of GVHD. The severity of GVHD was assessed using a clinical GVHD scoring system based on the reports of Cooke (45) and Schutt (46), with minor modifications. Briefly, mice were individually scored for the following 6 clinical parameters twice a week on a scale from 0 to 2: posture, activity, fur, alopecia, skin indentation (0, normal; 1, a small number of indentations on the back; 2, indentations over the whole body), and diarrhea (0, no diarrhea; 1, intermittent diarrhea; 2, continuous diarrhea). A clinical GVHD index was generated by summation of the 6 criteria scores, 0–12. Survival was monitored daily. For histopathological analyses of GVHD, target organs, colon, and skin tissues were formalin-preserved, paraffin-embedded, sectioned, and subjected to H&E or GFP staining. In particular, semiquantitative scoring for the intestine was performed by assessing the following findings (47): villous blunting, crypt regeneration, surface erosion, ulceration, loss of the enterocyte brush border, crypt cell apoptosis, luminal sloughing of cellular debris, cell vacuolization, surface cell attenuation, lamina propria lymphocytic, and neutrocytic infiltration. Each score was defined as follows: 0, normal; 1, focal and mild; and 2, diffuse and severe. Scores were summed to yield an overall score.

Analysis of GVT effects *in vivo*. In the B6D2F1 GVHD model, 5×10^4 GFP⁺-P815 cells were injected into B6D2F1 (H-2^{b/d}) recipient mice on day 0 simultaneously with B6 (H-2^{b/b}) TCD-BM with or without T cells. P815 cells are a mastocytoma cell line (H-2^{d/d}; RIKEN) derived from a DBA/2 mouse (H-2^{d/d}), and GFP was transfected in our laboratory. Tumor cell growth was analyzed by flow cytometry of peripheral blood and the spleens on day 10, and histopathologic analyses of the livers on day 9. In this model, P815 cells initially infiltrate the spleen and then the liver, BM, and spinal cord (33).

Flow cytometry. Single-cell suspensions of the peripheral blood, BM, and spleens were subjected to 6-color flow cytometry to detect surface antigens and intracellular phosphorylated ERK1/2. The antibod-

ies used for recognition of the specific antigens were anti-CD4 (GK1.5), anti-CD8 α (53-6.7), anti-CD19 (1D3), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-CD44 (IM7), anti-CD62L (MEL14), anti-H-2k^d (SF-1.1), anti-PD-1 (J43.1), anti-Lag-3 (C9B7W), and anti-Foxp3 (MF23) (Becton Dickinson); anti-Ly6G (1A8), anti-Ly6C (HK1.4), and anti-Tim-3 (RMT3-23) (BioLegend); anti-CD25 (PC61.5; eBiosciences); and anti-phospho-p44/42 MAPK (ERK1/2, 197G2) (Cell Signaling Technology). For staining phosphorylated ERK1/2 and Foxp3, fixation and permeabilization buffer sets (Life Technologies and Affymetrix) were used. Stained samples were assessed on a MACSQuant (Miltenyi Biotec) or a FACSVerse (Becton Dickinson) cytometer and analyzed using FlowJo Software (Tree Star).

Colony assay. BM cells from C57BL/6 mice were plated in MethoCult media (STEMCELL Technologies) at 37°C. DMSO or trametinib at 0.1 nM to 10 μ M was added, and the colony numbers of granulocytes, erythrocytes, and macrophages were counted on day 6. This experiment was performed in triplicate.

Cytotoxicity assay. Effector T cells (isolated with the MACS Pan T Cell Isolation Kit, Miltenyi Biotec) or NK cells (enriched with the NK Cell Isolation Kit, MACS; Miltenyi Biotec) from B6 (allogeneic) or DBA/2 (syngeneic) splenocytes were added to target CFSE-labeled P815 cells at various effector/target ratios in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc.). Plates were incubated for 4 hours with DMSO or 0.1 nM, 10 nM, or 1 μ M trametinib. Flow cytometric analyses were performed, and percentage lysis was calculated by dividing PI⁺CFSE⁺ cells by total CFSE⁺ cells.

Statistics. Statistical analyses and data presentation were performed using Prism software (GraphPad) on Mac computers (Apple). Data are expressed as mean \pm SEM. Statistical significance was defined as $P < 0.05$ using the 2-tailed unpaired Student's t test with or without Bonferroni correction or the log-rank test.

Study approval. The study was approved by the Saga University Institutional Animal Care and Use Committee as required.

Author contributions

HI performed the primary experiments, analyzed the data, and wrote the manuscript; TS designed the research, analyzed the data, and wrote the manuscript; IT contributed to the experimental design and manuscript editing; YK, RK, and SO helped to establish the experimental settings; KVK conceived the primary hypothesis and contributed to manuscript editing; and SK contributed to the research design and manuscript editing.

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