Sparing Regulatory T Cells

Disease by Inhibiting Effector T Cell Differentiation while ITK Selective Inhibitor Ameliorates Acute Graft-versus-Host

Pretransplant Short-Term Exposure of Donor Graft Cells to

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Pretransplant Short-Term Exposure of Donor Graft Cells to ITK Selective Inhibitor Ameliorates Acute Graft-versus-Host Disease by Inhibiting Effector T Cell Differentiation while Sparing Regulatory T Cells

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ABSTRACT
Graft-versus-host disease (GVHD) remains to be a significant cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). IL-2-inducible T cell kinase (ITK), a TEC cytoplasmic tyrosine kinase, has an essential role in T cell development and receptor signaling. The ITK/Bruton tyrosine kinase inhibitor ibrutinib has been shown to improve chronic GVHD symptoms; however, the effect of ITK selective inhibition on acute GVHD remains unclear. In this study, we evaluated the pharmacological effects of an ITK selective inhibitor (ITKsi) on acute GVHD using murine bone marrow transplantation models. First, we found that CD4+ T cell differentiation toward Th1, Th2, or Th17 was inhibited following ITKsi treatment in a dose-dependent manner while maintaining regulatory T cells in the presence of alloantigens both in vitro and in vivo. ITKsi preferentially inhibited inflammatory cytokine production and in vivo proliferation of alloreactive T cells. We then demonstrated that short-term exposure of donor graft cells to ITKsi significantly delayed the onset of GVHD-associated mortality without compromising the donor cell engraftment and the graft-versus-tumor effect, indicating the potential of ITK selective inhibition in the setting of clinical allogeneic HSCT. These findings suggest that ITK is a potential therapeutic target against GVHD, and the pharmacological ITK inhibitor may serve as a novel strategy for immune regulation after HSCT. ImmunoHorizons, 2021, 5: 424–437.

INTRODUCTION
Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective therapeutic strategy against various hematologic malignancies and congenital bone marrow disorders. Because of reduced-intensity conditioning regimens and alternative stem cell sources (1), the number of patients receiving allo-HSCT is increasing (2). However, graft-versus-host disease (GVHD) remains a major complication, causing nonrelapse mortality following hematopoietic stem cell transplantation (HSCT), particularly in cases of HLA-mismatched transplant, elderly recipients, and those bearing residual hematological disease at transplant (1, 2).
GVHD is triggered by donor-derived immune cells post-HSCT. Acute GVHD occurs within 100 days of HSCT and is characterized by clinical symptoms, such as skin rash, jaundice, and diarrhea. In contrast, chronic GVHD occurs in 30–70% of recipients and exhibits various clinical features resembling autoimmune and other immunologic disorders, such as scleroderma, Sjögren syndrome, primary biliary cirrhosis, wasting syndrome, bronchiolitis obliterans, immune cytopenia, and chronic immunodeficiency (3, 4).

Mature T cells in the donor inoculum play a major role in the pathogenesis of acute GVHD. The release of proinflammatory cytokines, alarmins, and pathogen-associated molecular patterns due to conditioning therapy induce maturation of donor T cells that become activated following interaction with host APCs in target organs, causing subsequent host tissue damage (5). Meanwhile, in chronic GVHD, both T and B cell immunity contribute to pathogenesis (6). However, both acute and chronic GVHD-associated pathology involve impaired recovery of regulatory T cells (Tregs), a CD4 T cell subset with broad immune-suppressive activity (7–9).

Although acute and chronic GVHD differ in their clinical characteristics and pathogenesis, recent studies have indicated that acute GVHD can trigger the pathogenesis of chronic GVHD (6, 10–12), suggesting that prevention of acute GVHD is critical for maintaining a high quality of life for long-term survivors (13, 14).

As prophylaxis for acute GVHD, a combination of calcineurin inhibitors and methotrexate has proven efficacious in most allo-HSCT recipients (6, 15). Calcineurin inhibitors reduce IL-2 transcription and activation of effector T cells while contributing to a concurrent reduction in IL-2-dependent Treg levels (16, 17). To address this, other immunosuppressive agents, such as mycophenolate mofetil and sirolimus, which do not interfere with IL-2 production and theoretically maintain the Treg population (16, 17), have undergone clinical trials showing promising results (18–20). However, several studies have reported a high prevalence of severe acute GVHD and vascular endothelial damage (15, 18–20). Thus, novel immunosuppressive modalities are warranted to reduce transplant-associated complications.

IL-2–inducible T cell kinase (ITK) (21), a member of the TEC family of tyrosine kinases primarily expressed in T cells, plays an essential role in naïve CD4 T cell development and TCR signaling. When the TCR is triggered, ITK is recruited to the plasma membrane (22) and activated by lymphocyte-specific protein tyrosine kinase (Lck) (23), which is important for the initiation of TCR signaling (24). Activated ITK phosphorylates phospholipase C-γ1 (PLC-γ1), thereby upregulating MAPK pathways and increasing intracellular Ca2+ levels, which in turn activate calcineurin, and ultimately activates NF of activated T cells (NFAT) (25). Meanwhile, the impact of ITK inhibition on Th polarization remains controversial. Previous studies, using Itk knockout mice, have demonstrated that Itk-deficient naïve CD4+ T cells suppress Th2 and Th17 differentiation without affecting Th1 (26–29) because resting lymphocyte kinase (RLK), a member of the TEC family, is involved in ITK signaling of Th1 cells (30–32). However, an Itk allele-sensitive mutant, the catalytic activity of which can be selectively inhibited by analogues of the PKC kinase inhibitor, showed that ITK activity is required not only for Th2 and Th17 cytokine production, but also for Th1 (33). In addition, recent studies have demonstrated that Itk-deficient CD4+ T cells, which are hypersensitive to IL-2 and readily differentiate into Tregs, help mitigate symptoms associated with autoimmune colitis in a mouse model (34).

The therapeutic efficacy of ITK selective inhibitors (ITKsi) has been previously investigated in several preclinical models. For instance, murine in vivo studies have demonstrated that ITK selective inhibition can ameliorate symptoms of multiple sclerosis (35) and psoriasis (36, 37) and prolong survival in a cardiac transplantation mouse model (38). In the context of allogeneic HSCT, a murine study showed that ibrutinib, a Bruton tyrosine kinase (BTK) and ITK multikinase inhibitor (39–41), suppresses the progression of bronchiolitis obliterans after allo-HSCT. A recent study showed that recipient mice transplanted with Itk knockout T cell grafts exhibited improved acute GVHD symptoms (42). However, the impact of pharmacological selective ITK inhibition of donor CD4+ T cells on acute GVHD remains unclear. ONO-7790500 is a new ITKsi that induced inhibition at an IC50 value <0.4 nM, compared with that of ibrutinib at 2.2 nM, and inhibited only three of 311 kinases, including ITK, at 0.3 μM (43–45). Moreover, ONO-7790500 reduced the population of human tonsillar CD4+ IL-17+ A+ cells and increased Foxp3+ cells in vitro (43). In this study, we examined the effects of an ITKsi, ONO-7790500, on acute GVHD using a murine bone marrow transplantation (BMT) model. Based on the results in this study, we propose that pharmacological inhibition of ITKs could be a novel strategy for the treatment of GVHD.

**MATERIALS AND METHODS**

**Mice**

Female C57BL/6 (CD45.2, H2Kb/b, B6) and B6D2Fl (CD45.2, H2Kb/b, BDF1) mice were purchased from CLEA Japan (Tokyo, Japan). Female BALB/c (CD45.2, H2Kb/b) mice were purchased from Japan SLC (Tokyo, Japan). Female C57BL/6 (CD45.1, H2Kb/b, Ly-5.1 B6) mice were purchased from the RIKEN Bio-Resource Center (Ibaraki, Japan). All mice were maintained under specific pathogen–free conditions and were 8–10 wk old throughout the study. The study protocols were reviewed and approved by the Animal Care and Use Committee, Okayama University Advanced Science Research Center (protocol number OKU-2018825). All experiments were performed in accordance with the study protocols. The study was carried out in compliance with the Animal Research: Reporting of In Vivo Experiments guidelines.

**CD4+ T cell isolation**

CD4+ T cells were isolated from the spleen of C57BL/6 mice using CD4 (L3T4) MicroBeads and an autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the
manufacturer's instructions. The purity of the CD4+ T cell fractions was greater than 95%.

Pretreatment of cells with ONO-7790500

CD4+ T cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS in the presence (pretreatment) or absence (control) of ONO-7790500 (donated from ONO Pharmaceutical, Osaka, Japan), as the ITKsi for 3 h. The cells were then washed twice with PBS containing 2% FBS.

Th cell polarization assay

Pretreated CD4+ T cells were incubated in a 96-well round-bottom plate at a concentration of 1 × 105 cells per well with 1 × 105 irradiated (30 Gy) peritoneal cells harvested from BALB/c and activated by anti-CD3ε (0.5 mg/ml; BD Biosciences) for 72 h in the presence of the following cytokine conditions: Th1-oriented conditions (IL-12, 10 ng/ml and anti–IL-4, 10 ng/ml); Th2-oriented conditions (IL-4, 10 ng/ml and anti–IFN-γ, 10 mg/ml); and Th17-oriented conditions (IL-6, 20 ng/ml, TGF-β, 5.0 ng/ml, IL-1β, 20 ng/ml, and anti–IFN-γ, anti–IL-4, and anti–IL-2, each at 10 ng/ml). Cells were plated for Th polarization for 72 h at 37°C.

Cell proliferation assay

Isolated CD4+ T cells were labeled with CellTrace Violet (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol. Labeled cells were transfused into recipient mice and splenocytes were harvested 3 d posttransfusion or plated and incubated for 72 h. Cell proliferation was analyzed using a MACSQuant (Miltenyi Biotec) flow cytometer.

Transplant procedure

On day 0, female B6D2F1 recipients were conditioned via lethal irradiation with two doses of 6 Gy, each administered 8 h apart. Recipient mice were injected with 1.5 × 106 splenic CD4+ T cells and 5 × 106 T cell–depleted bone marrow (TCD-BM) from donor mice on day 0. Donor CD4+ T cells were isolated from B6 spleen cells and treated ex vivo with 5000 nM ITKsi for 3 h. T cell depletion from donor bone marrow cells was conducted using anti–CD90.2 MicroBeads and an AutoMACS system (Miltenyi Biotec), according to the manufacturer’s instructions. To assess the graft-versus-tumor (GVT) effect, the P815 (H-2Kd) cell line was employed, which was established from a mastocytoma derived from a DBA/2 mouse. In a GVT model, B6D2F1 recipient mice received 8 Gy of total body irradiation (TBI) split into two doses. On day 0, the recipient mice were transplanted as described above, and 5 × 106 P815 cells were injected i.v.

Assessment of GVHD

Survival following HSCT was monitored daily, and the degree of clinical GVHD was assessed initially from day 3 to day 7 and subsequently once per week using a scoring system for five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10), as described previously (46).

RESULTS

ITKsi reduces CD4+ T cell differentiation toward Th1, Th2, and Th17 subsets in response to allogeneic Ags in vitro

To assess the potential effect of ONO-7790500 on CD4+ Th cell differentiation, we stimulated splenic CD4+ T cells from

Cytokine quantification

IFN-γ levels were quantified in culture supernatants using a Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocols. Multiplexed analysis of cytokines in mouse serum was performed using LEGENDplex Mouse Cytokine Kits (BioLegend). Data were collected on MACSQuant and analyzed using LEGENDplex software (BioLegend).

Flow cytometry

For transcription factor analysis, single-cell suspensions were first incubated with the following directly conjugated mAbs against various surface proteins (obtained from eBioscience, San Diego, CA, unless otherwise stated) for 20 min at 4°C: eFluor 450-conjugated anti-B220 (RA3-6B2), Brilliant Violet 510-conjugated anti–CD4 (GK1.5; BioLegend, San Diego, CA), allophycocyanin–eFluor 780–conjugated anti–CD8 (53-6.7), FITC-conjugated anti–H2Kd (SP1-11), PerCP-Cy5.5-conjugated anti–CD45.1 (A20), and PE-Cy7–conjugated anti–CD25 (PC61.5). Cells were processed for intracellular staining using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). The cells were incubated with the following for 30 min at 4°C: allophycocyanin-conjugated anti–Foxp3 (FJK-16s), Brilliant Violet 421-conjugated anti–GATA3 (3E10A23; BioLegend), PE-Cy7–conjugated anti–T-bet (4B10; BioLegend), and Alexa Fluor 647–conjugated anti–RORγt (Q31-378; BD Biosciences).

For intracellular cytokine analysis, differentiated cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (1.0 μg/ml; Sigma-Aldrich) for 4 h with the protein transport inhibitor GolgiPlug (1.0 mg/ml; BD Biosciences). Cells were first stained with FITC-conjugated anti–CD4 and subsequently fixed and permeabilized using a BD Cytofix/Cytoperm Kit (BD Biosciences). The cells were then incubated with allophycocyanin-conjugated anti–IFN-γ (XMGL1.2), PE-conjugated anti–IL-4 (1B11; BioLegend), or PC7-conjugated anti–IL-17A (TC11-18H10.1; BioLegend) for 30 min at 4°C. Samples were analyzed using a MACSQuant flow cytometer, and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

The results are presented as means ± SEM. The Mann–Whitney U test and Student t test were used to assess statistical significance between two groups, and one-way ANOVA was used to compare more than two groups. The Kaplan–Meier product limit method was used to determine survival probability, and the log-rank test was applied to compare survival curves. Statistical significance was set at p < 0.05, and all tests were two-tailed. GraphPad Prism 6 software (GraphPad, La Jolla, CA) was used for analysis.
FIGURE 1. ITKsi inhibits differentiation of CD4⁺ T cells toward Th1, Th2, and Th17 subsets in response to allogeneic Ags in vitro. (A) CD4⁺ T cells isolated from the spleens of B6 mice were cultured with BALB/c mouse peritoneal cells in the presence of ITKsi at the indicated concentrations in three different cytokine conditions: Th1-oriented conditions (IL-12, 10 ng/ml and anti-IL-4, 10 mg/ml), Th2-oriented conditions (IL-4, 10 ng/ml and anti-IFN-γ, 10 mg/ml) and Th17-oriented conditions (IL-6, 20 ng/ml; TGF-β, 5.0 ng/ml; IL-1β, 20 ng/ml; and anti-IFN-γ anti-IL-4, and anti-IL-2, each at 10 mg/ml). After 72 h, CD4⁺ T cells were analyzed for the expression of transcription factors (B and D) and the production of intracellular cytokines (C and E) specific to each Th polarization. Data from three independent experiments were compiled, and the data were normalized to the percentage of positive cells in the absence of an inhibitor. Data are expressed as the mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001. ONO, ONO-7790500.
B6 mice under Th1-, Th2-, or Th17-oriented conditions with peritoneal cells harvested from BALB/c mice as allogeneic Ags (Fig. 1A). Cells were cultured for 72 h in the presence of increasing doses of ONO-7790500 and subsequently analyzed for cytokine production after restimulation with PMA and ionomycin for 4 h. Th cell lineage-determining transcription factor analysis and intracellular cytokine analysis showed that ONO-7790500 inhibited IFN-γ production and decreased the expression of T-bet under Th1-oriented conditions in a dose-dependent manner. As with Th1-oriented conditions, ONO-7790500 inhibited IL-4 production and decreased the expression of GATA3 under Th2-oriented conditions and inhibited IL-17A production and decreased RORγt expression under Th17-oriented conditions (Fig. 1B-E). These results indicate that ONO-7790500 suppressed CD4⁺ T cell differentiation toward Th1, Th2, and Th17 cell subsets in response to allogeneic Ags.

Short-term ITKsi exposure reduces T cell differentiation toward Th1, Th2, and Th17 in response to allogeneic Ags in vitro

Next, we evaluated the responses of CD4⁺ T cells following short-term exposure to ONO-7790500. CD4⁺ T cells isolated from the spleens of B6 mice were cultured in the presence of ONO-7790500 at the indicated concentrations for 3 h. Cells were then washed and cultured under Th1-, Th2-, or Th17-oriented conditions with BALB/c peritoneal cells for 72 h (Fig. 2A). The pretreatment manipulation did not affect the viable cell rate (Supplemental Fig. 1); however, in vitro cell proliferation of pretreated CD4⁺ T cells was suppressed in a dose-dependent manner under each Th condition (Fig. 2B, 2C). Moreover, Th cell lineage-determining transcription factors were decreased upon pretreatment under each Th condition (Fig. 2D, 2E). These results suggest that pretreatment with ONO-7790500 can reduce CD4⁺ T cell division and differentiation under Th1-, Th2-, or Th17-oriented conditions.

Short-term ITKsi exposure reduces T cell proliferation and cytokine responses to allogeneic Ags in vitro

Pretreated CD4⁺ T cells, which were isolated from C57BL/6 mouse splenocytes, were cultured with BALB/c mouse peritoneal cells in the absence of any additional cytokines for 72 h (Fig. 3A). Results show that the proliferation of pretreated CD4⁺ T cells was suppressed to a greater extent than that of control cells (Fig. 3B, 3C). Moreover, the Treg population was larger in the pretreatment group compared with the control group (Fig. 3E). Furthermore, pretreatment reduced CD4⁺ T cell secretion of IFN-γ in culture supernatants in a dose-dependent manner (Fig. 3D).

Short-term ITKsi exposure reduces T cell proliferation in an acute GVHD mouse model

Based on the in vitro data, we assessed the allogeneic reaction following transplant of an ONO-7790500-pretreated graft in vivo. Lethally irradiated (12 Gy TBI) BDF1 mice were transplanted with 1.5 × 10⁶ splenic CD4⁺ T cells pretreated with 5000 nM ONO-7790500 from Ly-5.1 B6 mice (Fig. 4A). On day 3 after HSCT, mice were sacrificed, and the spleen was harvested. The in vivo division of donor graft-derived T cells, defined as CD45.1⁺ gated cells, was analyzed using flow cytometry. The rate of cell division was significantly lower in the pretreatment graft group compared with the control group on day 3 post-HSCT (Fig. 4B, 4C). Notably, the absolute number of graft-derived T cells was significantly lower in the pretreatment graft group than in the control group on day 3 (Fig. 4D).

**Pretreatment of donor graft cells with short-term ITKsi exposure delayed the development of acute GVHD and prolonged the overall survival**

Next, we evaluated the effect of transplanting a graft pretreated with ONO-7790500 on the development of acute GVHD using a murine model. Lethally irradiated (12Gy TBI) BDF1 mice were transplanted with 1.5 × 10⁶ splenic CD4⁺ T cells pretreated with 5000 nM of ONO-7790500 or control splenic CD4⁺ T cells from CD45.2 B6 mice and 5 × 10⁶ TCD-BM from CD45.1 B6 mice (Fig. 5A). Syngeneic transplants (BDF1→BDF1) were performed using the same procedure. Compared with the syngeneic model, the allogeneic model exhibited significantly inferior survival following HSCT. Furthermore, the allogeneic control graft group showed a markedly lower rate of survival than the allogeneic pretreatment graft group (Fig. 5B). Clinical GVHD scores in the allogeneic control group were significantly higher than in the pretreatment group after day 28 (Fig. 5C). ITK inhibition efficiently suppressed the GVHD scores, especially in the first month; however, the scores elevated gradually thereafter. We further confirmed GVHD amelioration by ONO-7790500 in another acute GVHD mouse model using B6 donors and BALB/c recipients (Supplemental Fig. 2A–C).

Furthermore, on day 7 after HSCT, serum cytokine assays demonstrated that TNF-α and IL-2 were significantly reduced in the pretreatment allograft group compared with the control group (Fig. 5D). In addition, serum IFN-γ levels were significantly higher in the allogeneic control group than in the syngeneic control group; however, no significant difference was observed between the allogeneic control group and the syngeneic control group. These results indicate that, similar to the in vitro data, pretreatment with ONO-7790500 suppressed graft CD4⁺ T cell division and inflammatory cytokine production in the early phase of allo-HSCT. As a result, pretreatment of CD4⁺ T cells led to amelioration of acute GVHD symptoms.

To verify the effect of ITKsi under conditions closer to clinical transplantation, we conducted the experiment with whole spleen cells containing various mature lymphocytes as transplanting grafts. Lethally irradiated (12Gy TBI) BDF1 mice were transplanted with 10 × 10⁶ whole spleen cells pretreated with 5000 nM ONO-7790500 and 5 × 10⁶ TCD-BM from B6 mice, with significantly superior survival compared with transplanted control spleen cells (Supplemental Fig. 3A–C).

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FIGURE 2. Short-term ITKsi exposure reduces T cell differentiation toward Th1, Th2, and Th17 in response to allogeneic Ags in vitro.

(A) CD4\(^+\) T cells isolated from spleens of B6 mice were cultured in the presence of ITKsi at the indicated concentrations for 3 h. Cells were then washed and cultured for 72 h with BALB/c mouse peritoneal cells in three different cytokine conditions, as indicated in Fig. 1. After 72 h, the proliferative response was assessed in CD4\(^+\) T cells (B and C) and the expression of transcription factors (D and E) specific to each Th polarization. Data from three independent experiments were compiled, and the data were normalized to the percentage of positive cells in the absence of an inhibitor. Data are expressed as the mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001.

ONO: ONO-7790500.
Pretransplant short-term exposure of donor graft cells to ITKsi maintains the GVT effect and does not impact B and T cell reconstitution

To evaluate GVT activity induced by pretreatment of grafts with ONO-7790500, we transferred luciferase-expressing P815 cells to acute GVHD model mice (Fig. 6A). In the syngeneic model, the tumor mortality rate increased in proportion to the onset of tumor-specific clinical features, such as leg paralysis or macroscopic tumor infiltration of the liver and spleen approximately 2 weeks after BMT. Meanwhile, tumor-free survival...
FIGURE 4. Short-term ITKsi exposure reduces T cell proliferation in vivo.
(A) CD4$^{+}$ T cells isolated from spleens of B6 mice were cultured in the presence or absence of 5000 nM ITKsi for 3 h. Cells were then adaptively transferred to irradiated B6D2F1 mice. Cell division was evaluated using the CFSE dilution assay in vivo on day 3. Recipients were sacrificed, and spleen cells were harvested. Representative image and summary data of cell division of graft-derived CD4$^{+}$ T cells (B and C), and the number of graft-derived CD4$^{+}$ T cells (D) are shown. Data from two independent experiments were combined. Data are expressed as the mean ± SE. **p < 0.01, ***p < 0.001.
FIGURE 5. Pretreatment of donor graft cells with short-term ITKsi exposure delayed the development of acute GVHD and prolonged the overall survival.

(A) Experimental scheme: Lethally irradiated (12 Gy TBI) B6D2F1 mice received transplants with $5 \times 10^6$ TCD-BM from B6 mice together with $1.5 \times 10^6$ 5000 nM ITKsi-pre-exposed CD4+ T cells ($n = 14$) or nontreated CD4+ T cells ($n = 10$) from B6 mice. The syngeneic group was administered the same dose of CD4+ T cells and TCD-BM from B6D2F1 mice ($n = 3$ per group). (B) Recipient survival is shown. Data from two independent experiments were combined. (C) Clinical scores of these recipients are shown as the mean ± SE. (D) Cytokine levels in plasma were measured using LEGENDplex on day 7. Data are representative of two independent experiments. Data are expressed as the mean ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. The asterisk (*), dagger (†), and double dagger (‡) symbols indicate the comparison between allogeneic control versus allogeneic pretreatment, syngeneic control versus allogeneic control, and syngeneic control versus allogeneic pretreatment, respectively.

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was significantly prolonged in the allogeneic model, demonstrating a significant GVT effect. Remarkably, in the allogeneic model, cumulative mortality rates for the control and pretreatment graft groups were comparable (Fig. 6B). However, on day 28 after BMT, flow cytometric analysis of splenocytes revealed that there was no significant difference in the number of B cells, CD8$^+$ T cells, Tregs, or CD4$^+$ T cells between the control and pretreatment groups (Fig. 6C). In addition, flow cytometric analysis of splenocytes showed that lethally irradiated (12 Gy TBI) B6D2F1 mice that were transplanted with $5 \times 10^6$ 5000 nM pretreated TCD-BM converted into donor chimerism on day 28 after BMT (Supplemental Fig. 4A–C).
DISCUSSION

Although previous studies have shown that ITK inhibition can ameliorate the symptoms of autoimmune diseases, including asthma (47, 48), multiple sclerosis (35), and psoriasis (36, 37), and prolong survival in a cardiac transplantation mouse model (38), the impact on acute GVHD in allogeneic HSCT has not been well studied. In the current study, we demonstrated that pharmacologic selective ITK inhibition modulates allogeneic T cell responses and prevents the development of acute GVHD in a murine BMT model. Our data suggest that ITK may represent a therapeutic target for the prevention of GVHD in allogeneic BMT settings.

First, we investigated the in vitro effects of ONO-7790500 on CD4⁺ T cells under each cytokine condition favoring the differentiation toward Th1, Th2, and Th17 cells. The analysis showed that expression of T-bet, GATA3, and RORγt following culture under Th1-, Th2-, and Th17-oriented cytokine conditions, respectively, were uniformly suppressed by ONO-7790500 in a dose-dependent manner (Fig. 1B, 1D). Dose-dependent suppressive effects were also observed in the expression of intracellular IFN-γ (Fig. 1B, 1D). The results indicate that CD4⁺ T cells were inhibited from dividing and readily differentiated into Th1 cells with increased T-bet expression and GATA3, and RORγt expression (Fig. 1B, 1D). These results suggest that CD4⁺ T cells exposed to sufficient concentrations of ONO-7790500 inhibit subsequent differentiation and cell division.

Next, to assess the response of ONO-7790500-treated CD4⁺ T cells to TCR and alloantigen stimulation, these cells were cultured without the addition of cytokines. Results show that pretreated CD4⁺ T cells were inhibited from dividing and readily differentiated into Tregs with decreased release of IFN-γ (Fig. 3B-E).

A previous study demonstrated that Itk-deficient CD4⁺ T cells readily differentiated into Tregs because of their increased sensitivity to IL-2, which enhanced both the STAT5 and PI3K/mTOR pathways without suppressing PTEN, a negative regulator of PI3K by TCR stimulation (34). In line with these results, our study results show that the number of Tregs was maintained, whereas other activated T cell populations decreased in recipients of ITK inhibitor-treated allografts (Fig. 6C). Furthermore, although calcineurin inhibitors have the potential to impair Treg recovery, our data suggest that ITK inhibitors may compensate for weaknesses in Treg recovery.

Based on the in vitro results, we assessed the impact of ONO-7790500 on in vivo T cell proliferation following allogeneic stimulation. The adaptive transfer experiment showed that the proliferation of ITK inhibitor-pretreated CD4 T cells was suppressed compared with non-pretreated controls on day 3 after the transfer (Fig. 4A-D). This result suggests that ONO-7790500 suppresses the alloreactivity of CD4⁺ T cells in vivo as well as in vitro.

We then confirmed that the reduction of alloreactivity by pretreatment with ITK inhibitor resulted in the amelioration of acute GVHD. Recipient mice that were transfused with pretreated CD4⁺ T cells also exhibited suppressed donor graft cell division in the early phase after HSCT, as well as reduced IL-2, TNF-α, and other inflammatory cytokine production (Fig. 5D), whereas they demonstrated improved survival compared with those that did not receive CD4⁺ T cells (Fig. 5B, 5C). A previous study showed that Th1 cytokines secreted by alloreactive T cells are intimately involved in the pathogenesis of GVHD (51). Cytokine storms, particularly those including TNF-α and IL-1, in the early stages of transplantation are important contributors to the pathogenesis of GVHD (52). Our data suggest that graft manipulation by ONO-7790500 could reduce the cytokine pathogenesis associated with acute GVHD.

Finally, we assessed the impact of ITK inhibition on GVT activity and long-term immune reconstitution. Although clinical data indicate that a potent GVT effect is generally associated with GVHD (53), a clinical strategy capable of distinguishing GVT from alloimmune reactions, which drives clinical GVHD, remains unknown (54). As shown in Fig. 6B, tumor-free survival in the allogeneic control was significantly prolonged compared with the syngeneic control, confirming the GVT effect was exerted based on the allogeneic immune response in

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this experimental system. Furthermore, the tumor-free survival in the allogeneic control group and the allogeneic ITK inhibition group were comparable. These data indicated that the addition of ITK inhibition did not sacrifice the GVT effect. As for immune reconstitution, pretreatment did not impact the recovery of donor B cells or T cell subsets, including Tregs (Fig. 6C). We also transplanted ONO-7790500-pretreated TCD-BM cells without splenic mature T cells, and we demonstrated that pretreatment did not impact significant effects on donor engraftment (Supplemental Fig. 4A–C). Hence, considering the clinical relevance, it is important to confirm the maintenance of both the GVT effect and immune reconstitution following therapeutic ITK modulation.

The primary objective of this study was to elucidate whether ITK can serve as a therapeutic target for acute GVHD following HSCT. Murine and clinical studies have demonstrated that ibrutinib, an ITK/BTK dual inhibitor, ameliorates the symptoms of chronic GVHD (39–41). However, the significance of ITK and BTK in the context of acute GVHD was not, to our knowledge, previously reported, although studies have shown that itk/ibrutinib can improve acute GVHD, even when functional B cells are depleted from the transplanted graft (40). Another study showed that activated T cells can express BTK, whereas inhibition of BTK expression on donor T cells ameliorates acute GVHD (55). The results of these studies indicate that ibrutinib can directly influence T cells without the involvement of B cells; however, whether ibrutinib affects T cells in an acute GVHD setting remains unclear. A recent study showed that Itk knockout T cell grafts produced fewer inflammatory cytokines, decreased migration to GVHD target organs, and ameliorated acute GVHD (42). To the best of our knowledge, our study is the first to demonstrate that pharmacological inhibition of ITK on donor T cells can ameliorate acute GVHD. Our results further support the phenomenon of Itk knockout T cell graft in an HSCT model and provides an important step toward the clinical application of ITK inhibition in acute GVHD.

Meanwhile, ITK deficiency does not lead to decreased B cell numbers (56, 57). Furthermore, BTK plays an essential role in the BCR and the pre-BCR signaling pathway, which regulates B cell development, proliferation, survival, and function (58, 59). Because ITK selective inhibition does not directly act on BTK in donor B cells during the early stage following HSCT, this strategy may provide a novel strategy for immune regulation that can selectively modulate acute GVHD-responsible T cells without impairing the early posttransplant BTK activation that is required to restore B cell-mediated immunity and reduce the risk of infectious events and subsequent chronic GVHD (12, 60).

In the current study, we focused on the differentiation of CD4+ Th cells, and our data showed that pharmacological ITK inhibition uniformly suppressed the Th1-, Th2-, and Th17-differentiation of naive CD4+ T cells in response to allogeneic stimulation. However, the significance of pharmacological ITK inhibition in the alloreactivity of other cell subsets, including CD8+ T cells, is not well understood. We showed that pretreatment of whole splenocytes with ONO-7790500 reduced the severity of acute GVHD, although not to the same degree as that observed in the CD4+ T cell BMT system (Supplemental Fig. 3A–C). This may suggest that the effect of ITK inhibition on lymphocytes other than CD4+ T cells is less significant than that on CD4+ T cells. However, a recent study actually showed that Itk knockout CD8+ T cells produced lower levels of inflammatory cytokines via allogeneic responses and maintained GVT effects cause upregulating expression of Eomesoderm (42). Thus, a similar effect of ONO-7790500 on CD8+ T cells may be expected. For the clinical application, the effect of ITK inhibitor on CD8+ T cell-mediated GVT effects should be warranted.

By using ITKsi, we showed the potential of pharmacological donor graft manipulation as a reasonable strategy to suppress GVHD in allogeneic HSCT. A previous study demonstrated that the donor α1-antitrypsin (AAT) treatment improved the symptoms of GVHD in the recipients via changing donor cells cytokine profile on major and minor mismatch GVHD mouse model (61). Moreover, in the clinical trial, patients with steroid-resistant acute GVHD showed promising responses to AAT (62).

These studies and our current study similarly showed that pharmacological graft manipulation can modulate posttransplant donor immunity and ameliorate GVHD, suggesting the ex vivo immune-modulating strategy has a potential for clinical application.

In this study, we showed the GVHD-suppressing effect by ex vivo graft manipulation with ITK inhibitor. GVHD scores in the first month were efficiently suppressed as compared with the allogeneic control, resulting in prolonged overall survival (Fig. 5B, 5C). However, the score increased gradually thereafter, suggesting that graft manipulation alone with ITK inhibitor is not sufficient to induce long-term immune tolerance. In future studies, the continuous treatment by ITK inhibitor after transplant or the combination therapy with other immune-suppressive agents should be considered. Such further research would contribute to the promotion of the use of ITK inhibition in clinical HSCT.

DISCLOSURES

The authors have no financial conflicts of interest.

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Supplemental Fig. 1

Short-term ITKsi exposure does not increase abundance of apoptotic or necrotic cells. CD4+ T cells isolated from spleens of B6 mice were cultured in the presence of ITKsi at the indicated concentrations for 1, 3, or 6 h. The cells were first incubated with directly conjugated monoclonal antibodies and eFluor 780-conjugated Fixable Viability Dye (FVD) (Thermo Fisher Scientific) for 30 min at 4 °C. After 30 min of incubation, the cells were washed twice and incubated with PE-conjugated AnnexinV(Thermo Fisher Scientific) for 15 min at room temperature. The following procedures were same described in the main part. Annexin-V+ and FVD− cells were defined as apoptotic, and Annexin-V− and FVD+ cells were defined as necrotic.
Supplemental Fig. 2

Pretransplant short-term exposure of donor graft cells to ITKsi ameliorates acute GVHD symptoms in another BMT model. (A) Experimental scheme: lethally irradiated (8Gy TBI) BALB/c mice received transplants with 5 × 10^6 TCD-BM from B6 mice together with 1.0 × 10^6 5000 nM ITKsi-pre-exposed CD4^+ T cells for 3 hours or non-treated CD4^+ T cells from B6 mice (n = 7/group). The syngeneic group was administered the same dose of CD4^+ T cells and TCD-BM from BALB/c mice (n = 2/group). (B) Recipient survival is shown. (C) Clinical scores of these recipients are shown as the mean ± SE. *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001. *, †, and ‡ indicate the comparison between allogenic control vs allogenic pretreatment, syngeneic control vs allogenic control, and syngeneic control vs allogenic pretreatment, respectively.
Supplemental Fig. 3

(A) Experimental scheme: lethally irradiated (12Gy TBI) B6D2F1 mice received transplants with $5 \times 10^6$ TCD-BM from B6 mice together with $10 \times 10^6$ 5000 nM ITKsi-pre-exposed whole spleen cells for 3 hours ($n = 15$) or non-treated spleen cells ($n = 14$) from B6 mice. (B) Recipient survival is shown. (C) Clinical scores of these recipients are shown as the mean ± SE. Data from two independent experiments were combined. * $p < 0.05$, ** $p < 0.01$

Pretransplant short-term exposure of donor graft spleen cells to ITKsi ameliorates acute GVHD.
Supplemental Fig. 4

**Pretransplant short-term exposure of donor stem cells to ITKsi does not affect engraftment.** (A) Experimental scheme: Lethally irradiated (12Gy TBI) B6D2F1 (CD45.2) mice received transplants with $5 \times 10^6$ 5000nM ITKsi-pre-exposed TCD-BM alone, or non-treated TCD-BM alone from Ly5.1 B6 (CD45.1) mice (n = 3/group). All mice were sacrificed and spleen was harvested on day 28 after HSCT. (B) Absolute number of splenocytes. (C) Flow cytometric analysis of splenocytes revealed chimerism. After Lymphocytes were gated in an FSC vs. SSC dot plot, CD45.1$^+$ cells were defined as donor-derived cells and CD45.1$^-$ cells were defined as host-derived.