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Gilteritinib enhances graft-versus-leukemia effects against FLT3-ITD mutant leukemia after allogeneic hematopoietic stem cell transplantation

Zixuan Zhang^{1*}, Yuta Hasegawa^{1*}, Daigo Hashimoto¹, Hajime Senjo¹, Ryo Kikuchi¹,
Xuanzhong Chen¹, Kazuki Yoneda¹, Tomoko Sekiguchi¹, Tatsuya Kawase², Hirofumi
Tsuzuki², Takashi Ishio¹, Takahide Ara¹, Hiroyuki Ohigashi¹, Masao Nakagawa¹,
Takanori Teshima¹

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

²Drug Discovery Research, Astellas Pharma Inc.,

* These authors contributed equally to this work

Corresponding author: Daigo Hashimoto, M.D., Ph.D.

N15 W7, Kita-ku, Sapporo 060-8638, Japan

Tel: +81-11-706-7214; Fax: +81-11-706-7823

E-mail: D5hash@pop.med.hokudai.ac.jp

Abstract

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a potentially curative therapy for FLT3 internal tandem duplication mutant (FLT3-ITD⁺) acute myeloid leukemia, but relapse rate is high. A recent study showed that sorafenib, a first generation FLT3 and multikinase inhibitor, enhanced graft-versus-leukemia (GVL) effects against FLT3-ITD⁺ leukemia via interleukin-15 (IL-15) production. However, it remains to be clarified whether this effect could be mediated by selective FLT3 inhibition. We investigated whether gilteritinib, a selective FLT3 inhibitor, could enhance GVL effects against FLT3-ITD transfected Ba/F3 leukemia (Ba/F3-FLT3-ITD) in mice. Oral administration of gilteritinib from day +5 to +14 after allo-SCT reduced expression of the co-inhibitory receptors PD-1 and TIGIT on donor CD8⁺ T cells and enhanced IL-15 expression in Ba/F3-FLT3-ITD. Bioluminescent imaging using luciferase-transfected Ba/F3-FLT3-ITD demonstrated that gilteritinib significantly suppressed leukemia expansion after allo-SCT, whereas it did not impact the morbidity or mortality of graft-versus-host disease (GVHD), resulting in significant improvement of overall survival. In conclusion, short-term administration of gilteritinib after allo-SCT enhanced GVL effects against FLT3-ITD⁺ leukemia without exacerbating GVHD.

Introduction

FMS-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD), which is attributed to a head-to-tail duplication of 3-400 base pairs in the juxtamembrane-domain coding sequence, is the most frequent (20-25%) mutation in adult acute myeloid leukemia (AML)^{1, 2}. The FLT3-ITD mutation is associated with a high leukemic burden and a poor prognosis of AML³⁻⁵. Although allogeneic hematopoietic stem cell transplantation (allo-SCT) potentially improves the outcomes of FLT3-ITD⁺ AML, the relapse rates are significantly higher in FLT3-ITD⁺ AML than those in FLT3-ITD negative AML after allo-SCT⁶⁻¹⁰.

FLT3 inhibitors are being developed to target constitutively activated FLT3-ITD signaling, which induces the proliferation and survival of leukemia cells. First-generation FLT3 inhibitors, such as sorafenib, are not specific to FLT3 and are categorized as multikinase inhibitors, whereas the second-generation FLT3 inhibitors, such as gilteritinib and quizartinib, are more specific to targeting mutated FLT3. In murine models of allo-SCT, sorafenib has been shown to induce the interferon regulatory factor 7 (IRF7)-dependent production of IL-15 in leukemia cells, which promotes the expansion of donor cytotoxic T cells and potentiates graft-versus-leukemia (GVL) effects against FLT3-ITD⁺ leukemia⁵. However, it remains to be clarified as to

58 whether second-generation FLT3 inhibitors can promote GVL effects after allo-SCT. In
59 the current study, we explored the impacts of the short-term administration of
60 gilteritinib on GVHD and GVL effects after mouse allo-SCT.

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Materials and methods

Mice: Female C57BL/6 (H-2^b) and B6C3F1 (H-2^{b/k}) mice were purchased from CLEA Japan (Shimizu, Japan) and Charles River Japan (Kanagawa, Japan), respectively. Mice were 8-12 weeks of age at the time of transplantation and were maintained in a specific pathogen-free environment. The recipient mice were randomly allocated to each experimental group, thus ensuring that the mean body weight in each group was similar. All the animal experiments were performed in a nonblinded fashion and under the auspices of the Institutional Animal Care and Research Advisory Committee.

Cell line: FLT3-ITD transfected C3H-derived Ba/F3 leukemia cells (Ba/F3-FLT3-ITD), FLT3-ITD-untransfected Ba/F3, and human myeloid leukemia cell line harboring FLT3-ITD (MOLM-13 cells) were provided by Astellas Pharma Inc. (Tokyo, Japan)¹¹. C57BL/6-derived myeloid leukemia cell line C1498 was purchased from ATCC. To create a luciferase expressing Ba/F3-FLT3-ITD cell line (Ba/F3-FLT3-ITD-luc⁺), the retroviral vector pMSCV-luc-IRES-YFP was kindly provided by Dr. Gerard Grosveld (Department of Genetics, St. Jude Children's Research Hospital). Briefly, Ba/F3-FLT3-ITD cells were infected with virus particles generated from HEK293T cells that were transfected with pMSCV-luc-IRES-YFP plasmid and pCL-Eco (Addgene, Cambridge, MA) in the RPMI 1640 medium supplemented with 1 µg/ml puromycin

(Sigma-Aldrich Japan, Tokyo, Japan) and 6 µg/ml polybrene (Nakarai Tesque, Kyoto, Japan)¹². pCL-Eco was obtained from Dr. Inder Verma (Addgene plasmid #12371)¹³. After infection, a single YFP expressing clone was recovered by using the limiting dilution method.

SCT: On day 0, B6C3F1 (H-2^{b/k}) recipients were lethally irradiated with 10.5 Gy total body irradiation, and split into 2 doses with 4 hr intervals, followed by i.v. injection with 5×10^6 T-cell depleted BM (TCD-BM) cells either alone or in combination with 1×10^6 purified T cells from major histocompatibility complex haploidentical B6 (H-2^b) on day 0. The purification of T cells and TCD-BM was performed by using Pan T Cell Isolation Kit II, mouse (Miltenyi Biotec Japan, Tokyo, Japan) and anti-CD90-MicroBeads (Miltenyi Biotec Japan), respectively, as well as and the AutoMACS Pro Separator (Miltenyi Biotec Japan). To assess the GVL effects, recipient mice were transferred with 5×10^4 or 5×10^5 Ba/F3-FLT3-ITD-luc⁺ cells on day 0. Gilteritinib, which was provided by Astellas Pharma Inc., was dissolved in 0.5% methylcellulose at a concentration of 1.25 mg/ml, and recipient mice were orally administered with either gilteritinib at 10 mg/kg/day or vehicle from day +5 to day +14 after allo-SCT.

Evaluation of GVHD and GVL: Survival was monitored daily, and the severity of

GVHD was assessed by using the GVHD scoring system with five parameters¹⁴.

Bioluminescent imaging (BLI) was conducted weekly to evaluate the GVL effects. Mice

were anesthetized by using isoflurane and were subcutaneously injected with 500 µg

d-luciferin (Promega, Madison, WI) at 5 min before *in vivo* imaging. Luciferase⁺ cells

were detected by using IVIS Imaging System ver. 4.3.1 (Perkin Elmer, Waltham, MA).

Light emission is presented as photons per second per square centimeter per ster

adian (ph/s/cm²/sr). Leukemia death was defined by the existence of

hepatosplenomegaly with macroscopic tumor nodules, hinder leg paralysis¹⁵, or

leukemia expansion on BLI. Deaths without these signs were attributed to GVHD. To

evaluate allospecific cytotoxic T lymphocyte (CTL) responses, vehicle- or

gilteritinib-treated recipients of TCD-BM plus T cells were i.v. injected with 3×10^6

untransfected Ba/F3 (allogeneic) or C1498 (syngeneic) labeled with 5 µM CellTraceTM

Violet (CTV, Thermo Fisher Scientific) on day +8 after allo-SCT. CTV positive cells in

the bone marrow were enumerated 2 hrs after the transfer.

Culture: Ba/F3-FLT3-ITD cells or MOLM-13 cells (5×10^5 cells/well) were cultured in

RPM I1640 medium containing 10% FCS in 6-well plates. Cells were stimulated with

either gilteritinib or quizartinib (Selleck Biotech, Tokyo, Japan) at concentrations of 1.0,

10, or 100 nM, or sorafenib (SYNKinase, Shanghai, China) at concentrations of 0.1, 1.0,

or 10 μ M for 24 hrs.

Q-PCR: The total RNA extracted from the leukemia cells by using either ISOGEN II (Nippon Gene, Toyama, Japan) or RNeasy Kit (QIAGEN) was subjected to cDNA synthesis by using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo FSQ-301, Osaka, Japan) or SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Quantitative PCR (Q-PCR) was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Redmond, WA) by using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA) or on the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific), with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and the following primer-probe sets. The mouse 18S rRNA primer probe set (Sigma Aldrich) was 5'-GCTCTTTCTCGATTCCGTGGG-3' for the forward primer, 5'-ATGCCAGAGTCTCGTTTCGTTATC-3' for the reverse primer, and FAM-CTCCACCAAC TAAGAACGGCCATGCACC-TAMRA for the probe, or 4319413E set (Thermo Fisher Scientific). The human GAPDH primer probe set was 4319413E set (Thermo Fisher Scientific). The IL-15 primer-probe sets were Mm00434210_m1 (Thermo Fisher Scientific) for mouse cells and RT² qPCR Primer Assay PPH00694B (QIAGEN) for human cells. The expression levels of 18S rRNA or

GAPDH were used as a standard.

Flow cytometric analysis: The monoclonal antibodies (mAbs) that were used were PE-CF594-, PE-Cy7-, PerCP-Cy5.5-, APC-, BV510-, BV605-, or BV786-conjugated anti-mouse CD4 (Cat#: 553051), CD8 (Cat#: 563152), CD44 (Cat#: 563114), CD62L (Cat#: 560516), TCR β (Cat#: 560657), TIGIT (Cat#: 744215), and PD-1 (Cat#: 562523) purchased from BD Pharmingen (San Diego, CA). The dead cells were excluded from the analyses, based on the positivity of the Fixable viability stain 780 from BD Pharmingen. Cells were analyzed by using a FACS Aria III (BD Bioscience, Tokyo, Japan) and FlowJo software (BD Biosciences).

Statistical analysis: Dunnett's multiple comparison test was used to compare *in vitro* IL-15 expression, and Mann-Whitney U tests were used to compare other data. The Kaplan–Meier product limit method was used to obtain survival probability and the log-rank test was applied to compare the survival curves. Cumulative incidences of leukemia or GVHD death in each experimental group were compared by using the Gray's test. Analyses were performed by using GraphPad Prism 8 software (GraphPad Software, San Diego, CA) and EZR (Saitama Medical Center, Jichi Medical University, Japan), the graphic user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)¹⁶. Results with $P < 0.05$ were statistically significant.

Results

Gilteritinib promotes IL-15 production from FLT3-ITD⁺ leukemia

First, we tested whether gilteritinib could stimulate FLT3-ITD-expressing leukemia cells to produce IL-15. Ba/F3-FLT3-ITD cells and human myeloid leukemia cells harboring FLT3-ITD (MOLM-13) were cultured in the presence or absence of gilteritinib, quizartinib, or sorafenib for 24 hrs. All three reagents significantly upregulated IL-15 expression both in Ba/F3-FLT3-ITD and MOLM/13 cells in a partially dose-dependent manner (**Figure 1, A and B**). To confirm these findings *in vivo*, lethally irradiated B6C3F1 mice were transplanted with 5×10^6 TCD-BM cells and 1×10^6 purified T cells from allogeneic B6 donors combined with 1×10^5 YFP-labeled Ba/F3-FLT3-ITD cells. Gilteritinib at 10 mg/kg/day or vehicle was orally administered from day +5 to day +8 after allo-SCT. The Q-PCR analysis showed a significant upregulation of IL-15 in Ba/F3-ITD cells that were purified from the spleen and bone marrow of the gilteritinib-treated mice on day +8 (**Figure 1, C and D**). The flow cytometric analysis showed that gilteritinib significantly reduced the co-inhibitory receptors, PD-1 and TIGIT on donor CD8⁺ T cells in the spleen, thus suggesting that IL-15 production induced by gilteritinib inhibited T-cell exhaustion after allo-SCT (**Figure 1, E to G**). To evaluate allospecific donor CTL responses, vehicle- or

gilteritinib-treated recipients of TCD-BM plus T cells were i.v. injected with CTV-labeled untransfected Ba/F3 (allogeneic) or C57BL/6-derived C1498 (syngeneic) cells on day +8 after allo-SCT, and CTV⁺ leukemia cells were enumerated in the bone marrow 2 hrs later. We found that untransfected Ba/F3 cells were significantly reduced in gilteritinib-treated mice compared to vehicle treated recipients, while the number of donor-derived C1498 were comparable in vehicle- and gilteritinib-treated recipients, indicating that gilteritinib enhanced allospecific donor CTL responses (**Figure 1, H and I**).

Gilteritinib enhances graft-versus-leukemia effects against FLT3-ITD-mutant leukemia

Next, we tested whether gilteritinib could enhance GVL effects against Ba/F3-FLT3-ITD cells after allo-SCT. Lethally irradiated B6C3F1 mice were transplanted with 5×10^6 TCD-BM cells either alone or in combination with 1×10^6 purified T cells from allogeneic B6 donors. Recipient mice were transferred with 5×10^4 Ba/F3-FLT3-ITD-luc⁺ cells on day 0, and gilteritinib was administered from day +5 to day +14 after allo-SCT. The BLI demonstrated that gilteritinib had only modest anti-leukemic effects in the recipients of TCD-BM; all vehicle-treated and

gilteritinib-treated recipients of TCD-BM died with leukemia by day +15 and day +22, respectively (**Figure 2, A and B**). Leukemia death was significantly suppressed in the recipients of T cells compared to TCD-BM controls, thus indicating GVL effects. However, all the mice succumbed to leukemia or GVHD by day +46 (**Figure 2, A and F**). In GVHD mice, short-term administration of gilteritinib significantly suppressed the expansion of Ba/F3-FLT3-ITD-luc⁺ cells and reduced leukemia death after T-cell replete allo-SCT; 12 out of 15 vehicle-treated recipients and 8 out of 15 gilteritinib-treated recipients after T-cell replete allo-SCT succumbed to leukemia (**Figure 2A**). Cumulative incidence of leukemia death in gilteritinib-treated recipients was 60% at day +80 and significantly lower than that in vehicle-treated allogeneic recipients (**Figure 2B**). Importantly, gilteritinib did not impact the morbidity or mortality of GVHD, thus resulting in significantly prolonged overall survival in the recipients of T cells (**Figure 2, C to F**). Taken together, short-term gilteritinib administration enhanced GVL effects against FLT3-ITD⁺ leukemia without exacerbating GVHD.

Discussion

Emerging evidence has suggested that FLT3 inhibitor is more effective in the posttransplant setting than in the nontransplant setting¹⁷. The first-generation FLT3 and multi-kinase inhibitor, sorafenib has been shown to induce more long-lasting remission in patients after allo-SCT than in patients relapsing after chemotherapy¹⁸⁻²⁰. Recent randomized trials have demonstrated that sorafenib maintenance after allo-SCT significantly reduced the risks of relapse and death of FLT3-ITD positive AML^{21, 22}. These findings suggest that FLT3 inhibitors can enhance GVL effects in addition to their direct cytotoxic effects on FLT3-ITD-mutated AML cells. In preclinical models, sorafenib activates IRF7 by downregulating activating transcription factor 4 (ATF4), which enhances IL-15 production from leukemia cells⁵. IL-15 from leukemia cells has been shown to expand CD8⁺CD107a⁺IFN- γ ⁺ donor cytotoxic T cells with increased Bcl-2 and reduced PD-1 expression levels, which enhanced GVL effects against FLT3-ITD-positive leukemia⁵. Recently, it has been reported that leukemia-derived lactic acid impaired metabolic activity of T cells inducing dysfunctional T cells²³. Because metabolic reprogramming is also associated with T cell exhaustion, it is intriguing to evaluate if FLT3 inhibitors could modulate production of lactic acid in leukemia cells in the future studies²⁴.

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224 Despite the findings that support the GVL-potentiating effects of sorafenib, the
225 impacts of selective FLT3 inhibitors such as gilteritinib on GVL effects have not been
226 well studied. We herein demonstrated that the administration of gilteritinib after
227 allo-SCT enhances IL-15 production from mouse FLT3-ITD-positive leukemia,
228 potentiates CTL responses of donor T cells, and promotes GVL effects after allo-SCT.
229 Long-term suppression of leukemia growth was observed in some of the
230 gilteritinib-treated T-cell-replete recipients, thus suggesting that gilteritinib contributed
231 to an immune-mediated cure of FLT3-ITD-positive leukemia after allo-HCT. Although
232 we haven't tested if other selective FLT3 inhibitors such as quizartinib affect donor
233 T-cell profiles or GVL effects after allo-SCT, enhanced IL-15 expression in
234 quizartinib-treated leukemia cells suggests that FLT3 inhibitors in general can mitigate
235 exhaustion of donor T cells and enhance GVL effects after allo-SCT.

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237 IL-15 production from leukemia cells significantly reduces T-cell expression of
238 PD-1, which is a critical molecule for tolerance induction after allo-SCT^{25, 26}, which
239 raises the concern that FLT3-ITD inhibition can exaggerate GVHD. A randomized trial
240 showed that maintenance therapy with sorafenib after allo-SCT can reduce the risk of

relapse without increasing acute or chronic GVHD²², whereas there was a trend towards increased GVHD in another randomized trial²¹. It is worth noting that gilteritinib did not impact the clinical GVHD scores, body weight changes, or mortality of GVHD in our model, even though GVHD was more severe with shorter survival in our model, compared to the previous sorafenib study, in which the delayed infusion of donor T cells after allo-SCT mitigated GVHD, as has been previously shown^{5, 27, 28}. It has been suggested that sorafenib-treated leukemia cells produce both IL-15 and IL-15Ra and can trans-present the IL-15/IL-15Ra complex to donor T cells²⁹. Thus, FLT3-ITD inhibition may activate donor T cells localizing around leukemia cells, without activating T cells in the GVHD target organs. Furthermore, FLT3-ITD inhibitors likely activate immunity only in the presence of leukemia cells, because they require IL-15 production from leukemia cells. This nature of action of FLT3-ITD inhibitors makes FLT3-ITD inhibition after allo-SCT safer and enables us to elicit just enough immunity to eradicate leukemia cells.

It is important to assess whether gilteritinib reduced leukemia death by enhancing GVL effects or solely by direct anti-leukemia effects of FLT3 inhibition. We consider gilteritinib enhanced GVL effects for following reasons. First, BLI analyses

demonstrated that leukemia cells started to expand during gilteritinib-treatment in the recipients transplanted with TCD-BM alone (Figure 2A, day12), indicating that gilteritinib alone is not enough to reject Ba/F3-FLT3-ITD cells after T-cell deplete allo-SCT. Second, *in vivo* CTL assay demonstrated that gilteritinib significantly enhanced allospecific CTL responses against FLT3-ITD-untransfected Ba/F3. Therefore, we concluded that gilteritinib reduced leukemia death at least partially by enhancing GVL effects. We used short-term administration of gilteritinib to minimize the direct anti-leukemia effect against Ba/F3-FLT3-ITD.

In summary, short-term gilteritinib administration after allo-SCT promotes IL-15 production from FLT3-ITD-positive leukemia cells and enhances GVL effects without affecting GVHD severity. Our data showing that gilteritinib enhanced GVL effects support the use of gilteritinib as a maintenance. However, since gilteritinib effects require residual AML cells, it is unclear if maintenance is effective in the recipients with minimal amount of residual AML cells⁵. Thus, the safety and efficacy of gilteritinib administration after clinical allo-SCT need to be studied in future and ongoing clinical trials.

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Author contributions

D.H. and T.T. developed the conceptual framework of the study, designed the experiments, analyzed the data and wrote the paper. Z.Z and Y.H. conducted experiments, analyzed data, and wrote the paper. H.S., R.K., X.C, K.Y., T.S., T.K., H.T., T.I., T.A., and H.O. conducted experiments. M.N. supervised experiments.

Conflict of Interest

T.T. received a research grant from Kyowa Kirin, Chugai, Sanofi, Astellas, TEIJIN PHARMA, Fuji Pharma, NIPPON SHINYAKU, Personal Fees from Novartis, Merck, Kyowa Kirin, Takeda, Pfizer, Bristol-Myers Squibb, Non-Financial Support from

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Figure Legends

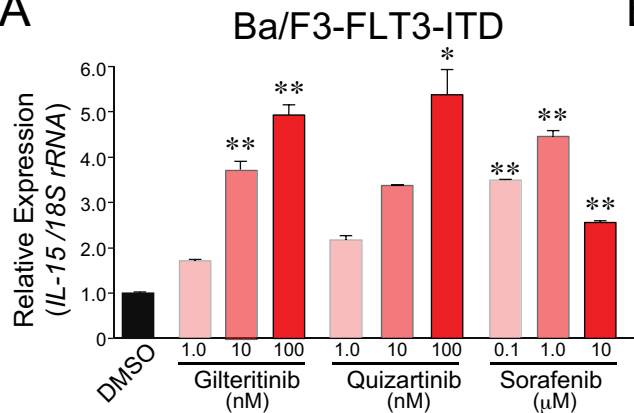
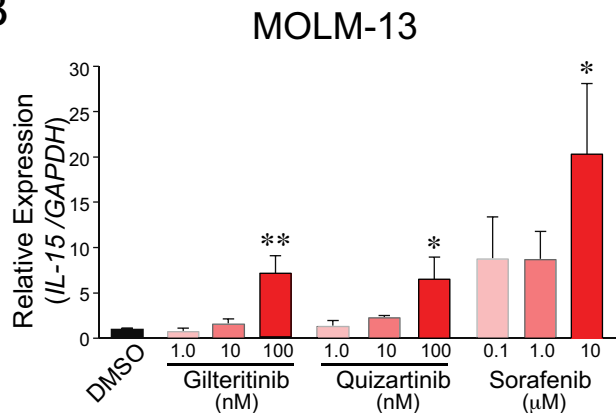
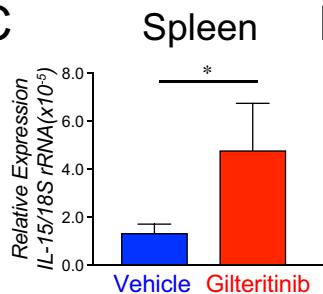
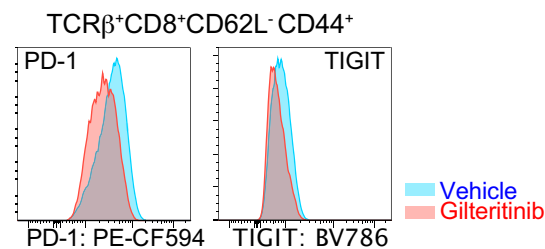
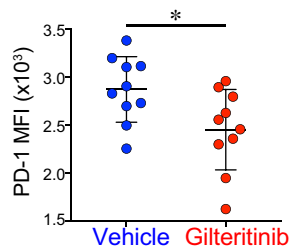
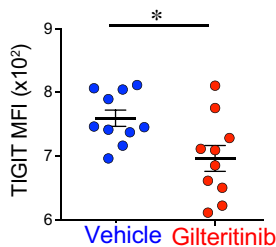
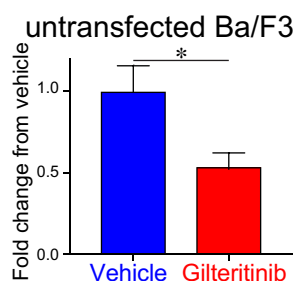
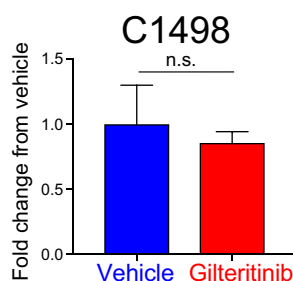
Figure 1. FLT3-ITD inhibitors promote IL-15 expression in Ba/F3-FLT3-ITD cells

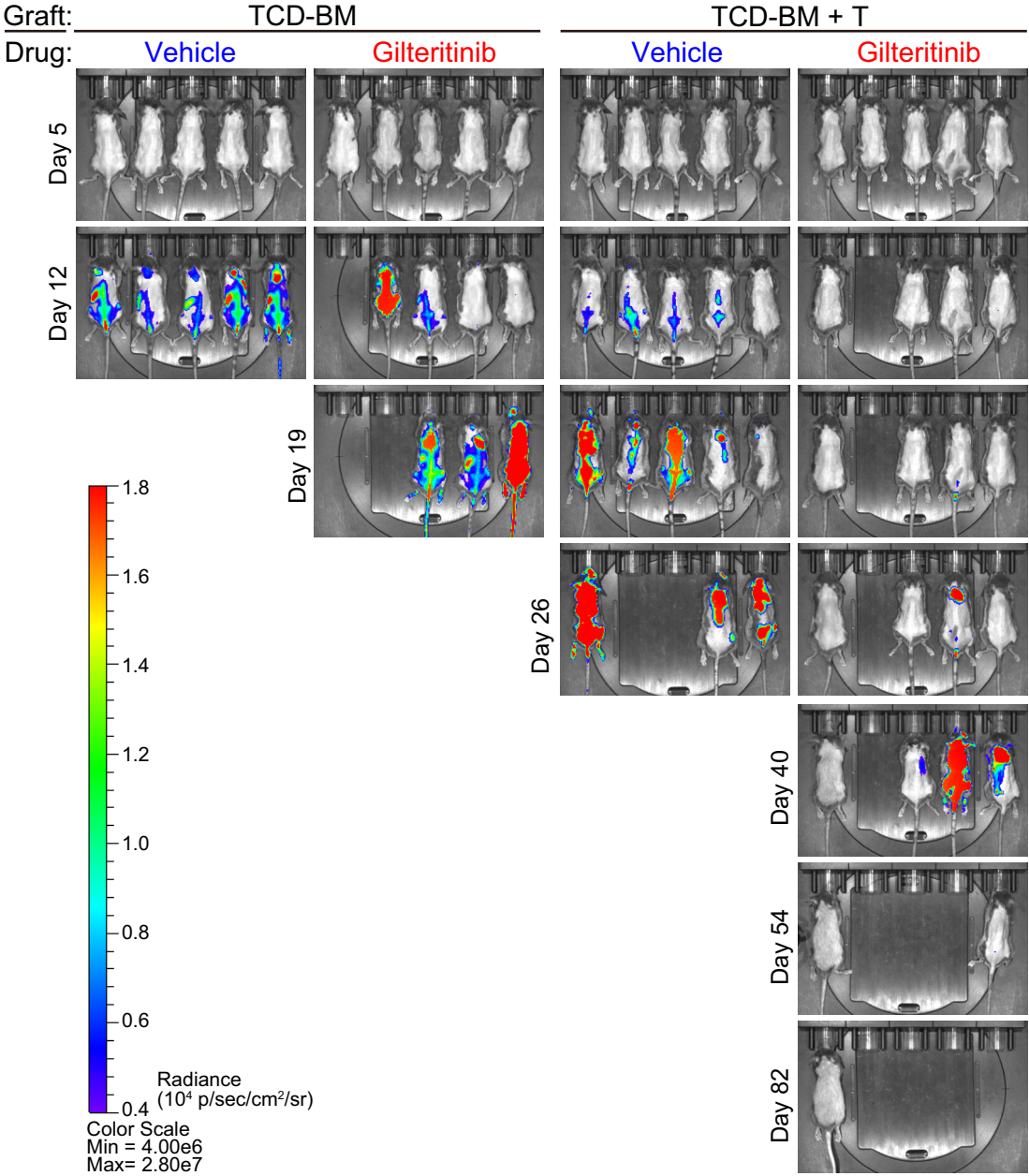
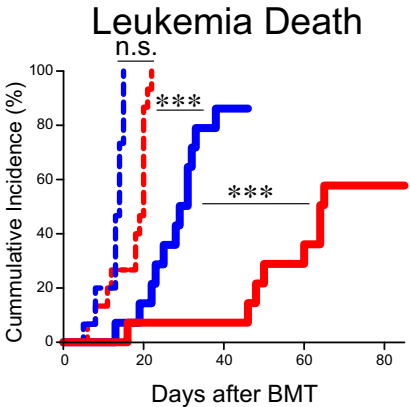
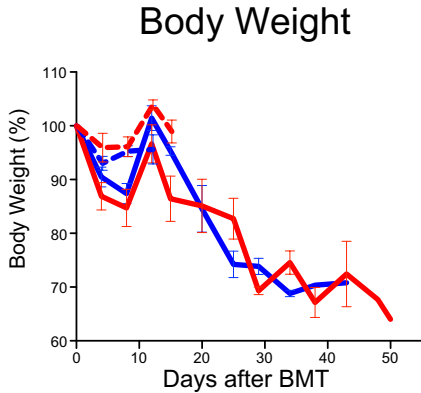
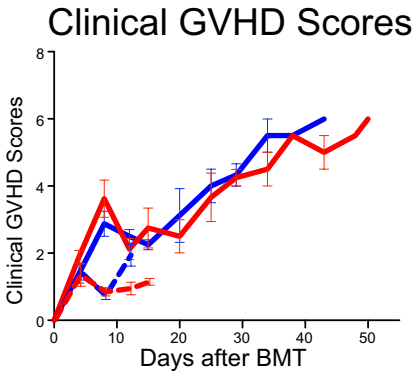
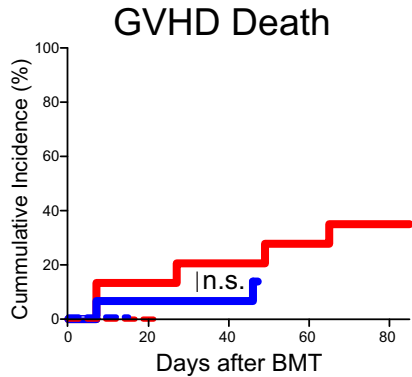
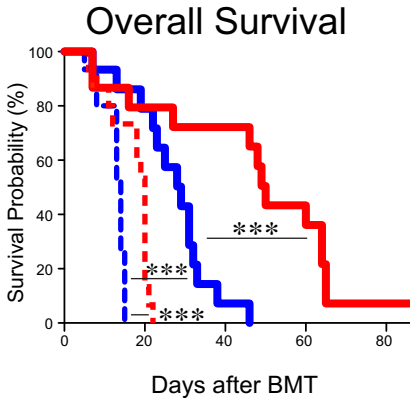
(A,B) Ba/F3-FLT3-ITD **(A)** or MOLM-13 **(B)** cells were cultured in the presence or absence of gilteritinib or quizartinib at concentrations of 1.0, 10, or 100 nM or sorafenib at concentrations of 0.1, 1.0, or 10 μ M for 24 hrs. RNA extracted from cells was subjected to qPCR targeting IL-15. The expression levels of IL-15 were normalized to 18S rRNA **(A)** or GAPDH **(B)** expression levels. Data were collected in triplicate and are shown as the means $\pm$ SDs. **(C-G)** Lethally irradiated B6C3F1 mice were transplanted with 5×10^6 TCD-BM cells and 1×10^6 purified T cells from allogeneic B6 donors combined with 1×10^5 Ba/F3-FLT3-ITD cells, followed by oral administrations of 10 mg/kg/day gilteritinib or vehicle from day +5 to day +8 after allo-SCT. **(C,D)** Relative expression levels of IL-15 in Ba/F3-FLT3-ITD cells purified from the spleen **(C)** and BM **(D)** on day +8 after allo-SCT (n = 14/group). Data from three independent experiments are combined and shown as the means $\pm$ SEs. **(D-F)** Flow cytometric analysis of spleens was performed on day +8 after allo-SCT. Histograms **(E)** and mean fluorescence intensities of PD-1 **(F)** and TIGIT **(G)** on donor TCR β^+ CD8 $^+$ CD62L $^-$ CD44 $^+$ cells. Data from two independent experiments are combined and shown as the means $\pm$ SEs (n = 10/group). **(H,I)** Vehicle- or

gilteritinib-treated recipients of TCD-BM plus T cells were i.v. injected with 3×10^6 CTV-labeled untransfected Ba/F3 (C, n=7/group) or C1498 (D, n=5/group) cells on day +8 after allo-SCT. CTV-labeled cells in the bone marrow were enumerated 2 hrs after the injection. Data from two independent experiments are combined. *, $P < 0.05$; **, $P < 0.01$.

Figure 2. Gilteritinib enhances GVL effects against Ba/F3-FLT3-ITD-luc leukemia

Lethally irradiated B6C3F1 mice were transplanted with 5×10^6 TCD-BM cells either alone or in combination with 1×10^6 T cells from allogeneic B6 donors and transferred with 5×10^4 Ba/F3-FLT3-ITD-luc⁺ cells on day 0. Recipients were orally administered with either gilteritinib or vehicle from day +5 to +14 after allo-SCT. Representative images of BLI (A), cumulative incidences of leukemia death (B), body weight (C, means $\pm$ SDs), clinical GVHD scores (D, means $\pm$ SDs), cumulative incidences of GVHD death (E), and overall survival (F) in the recipients of TCD-BM treated with either vehicle or gilteritinib, and in the recipients of TCD-BM plus T cells treated with either vehicle or gilteritinib (n=15/group). (A) Images from one of three similar experiments are shown. (B-F) Data from three independent experiments were combined.

A**B****C****D****E****F****G****H****I**

A**B****C****D****E****F**

--- TCD-BM + Vehicle
--- TCD-BM + Gilteritinib
--- TCD-BM + T + Vehicle
--- TCD-BM + T + Gilteritinib