The druggable transcription factor Fli-1 regulates T cell immunity and tolerance in graft-versus-host disease

Steven D. Schutt, … , Yaacov Ben-David, Xue-Zhong Yu

J Clin Invest. 2022;132(21):e143950. https://doi.org/10.1172/JCI143950.

Graphical abstract

Find the latest version:

https://jci.me/143950/pdf
Graft-versus-host disease (GVHD), manifesting as either acute (aGVHD) or chronic (cGVHD), presents significant life-threatening complications following allogeneic hematopoietic cell transplantation. Here, we investigated Friend virus leukemia integration 1 (Fli-1) in GVHD pathogenesis and validated Fli-1 as a therapeutic target. Using genetic approaches, we found that Fli-1 dynamically regulated different T cell subsets in allogeneic responses and pathogenicity in the development of aGVHD and cGVHD. Compared with homozygous Fli1-deficient or WT T cells, heterozygous Fli1-deficient T cells induced the mildest GVHD, as evidenced by the lowest Th1 and Th17 cell differentiation. Single-cell RNA-Seq analysis revealed that Fli-1 differentially regulated CD4⁺ and CD8⁺ T cell responses. Fli-1 promoted the transcription of Th1/Th17 pathways and T cell receptor-inducible (TCR-inducible) transcription factors in CD4⁺ T cells, while suppressing activation- and function-related gene pathways in CD8⁺ T cells. Importantly, a low dose of camptothecin, topotecan, or etoposide acted as a potent Fli-1 inhibitor and significantly attenuated GVHD severity, while preserving the graft-versus-leukemia (GVL) effect. This observation was extended to a xenograft model, in which GVHD was induced by human T cells. In conclusion, we provide evidence that Fli-1 plays a crucial role in alloreactive CD4⁺ T cell activation and differentiation and that targeting Fli-1 may be an attractive strategy for treating GVHD without compromising the GVL effect.

Introduction

Currently, the most effective treatment in the clinic for hematological malignancies including leukemia, lymphoma, and myeloma is allogeneic hematopoietic cell transplantation (allo-HCT). Donor bone marrow (BM) or peripheral blood (PB) lymphocytes directly recognize and kill malignant cells within the host, termed the graft-versus-tumor (GVT) effect or graft-versus-leukemia (GVL) effect. A detrimental side effect of allo-HCT that occurs in 30%-70% of transplant recipients is chronic graft-versus-host disease (cGVHD). cGVHD is the primary cause of late-stage transplant-related morbidity and mortality despite available prophylactic strategies and treatments (1, 2).

Targeting T cell pathways remains a promising area of investigation in GVHD therapeutics. One potential T cell candidate target relevant to both leukemia and the immune response that has not, to our knowledge, been studied in GVHD pathogenesis is the transcription factor Friend virus leukemia integration 1 (Fli-1). Although Fli-1 has been relatively understudied specifically in primary lymphocytes, especially T cells, it was indeed previously demonstrated that retroviral overexpression of Fli1 in T cell progenitor cells led to initiation of uncontrolled T cell proliferation and pre-T cell lymphoblastic lymphoma mediated by notch-1 receptor protein (NOTCH-1) mutations (3), and that T cells from germline heterozygous Fli1-deficient mice bearing the Fas mutation showed that Fli-1 was positively associated with the inflammatory factors CXCR3, IL-6, C16-ceramide, IL-17, and GM-CSF (4–8). These factors can also play important roles in GVHD pathogenesis (6, 9–13). A recent role for Fli-1 in regulating the CD8⁺ T cell response during infection and the antitumor response has also been identified, yet its role in primary CD4⁺ T cells remains largely elusive (14).

Cancer chemotherapeutics including camptothecin (CPT), the CPT analog topotecan (TPT), and etoposide (ETO) were shown to be potent Fli-1 protein inhibitors (15, 16). In these studies, CPT impaired tumor growth in multiple erythroleukemia cell lines in vitro and in Friend murine leukemia virus–induced (F-MuLV-induced) erythroleukemia in vivo (15, 17), whereas TPT reduced lupus nephritis and inflammatory factors in human renal cells (16). Additionally, multiple cancer types that are targeted with allo-HCT such as acute myeloid leukemia (AML), lymphomas, and other hematopoietic malignancies have been shown to express
high levels of Fli1 mRNA, suggesting that targeting Fli-1 in these cancer types could be beneficial in reducing their growth (18).

Despite these previous findings, to date there has been no research to our knowledge that directly implicates Fli-1 as a link between immune tolerance and anti-leukemia immunity in the context of allo-HCT. Furthermore, whether Fli-1 plays an important role in the CD4+ T cell response has not to our knowledge been addressed until the current study. Here, we used a genetic strategy to target Fli-1 activity specifically on T cells and determined the role of Fli-1 in experimental GVHD models. We then used known pharmacological agents with strong Fli-1-inhibitory activity in preclinical allo-HCT mouse models of acute GVHD (aGVHD) and cGVHD, as well as in a humanized xenograft model of GVHD, and showed that decreasing the expression or activity of Fli-1 may be an important translational concept for reducing the pathogenesis of GVHD without impairing the GVL response.

Results

Characteristics of Fli1 conditional-KO mice. The Fli1fl/fl mouse strain was previously shown to effectively reduce Fli-1 mRNA and protein levels via Cre-mediated recombination on the Tie2 (Tek) promoter (19). We verified the ability of the CD4 promoter–based Cre/loxP system to mediate effective Fli1 recombination in T cells. Using a PCR probe specific for the consensus loxP sites present near exons 3 and 4, where, as expected, we found complete deletion of the exon 4 loxP site in Fli1fl/flCD4Cre+ T cells compared with Fli1fl/flCD4Cre- T cells, which contained both loxP sites, but no CD4Cre recombinase expression, indicating that effective cell-specific Fli1 recombination occurred in the presence of CD4Cre (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI143950DS1). In this Cre/loxP system, the Fli1 exon 4 loxP site is cleaved, while the exon 3 loxP site is maintained after recombination (19). Furthermore, we found significantly reduced Fli1 mRNA levels in T cells from Fli1fl/flCre+ (Fli1fl/wt and Fli1fl/fl) mice compared with Fli1fl/flCre+ (Fli1fl/fl) controls (Supplemental Figure 1B).

Fli-1 is a signaling component known to be essential for IL-2 production as well as GVHD development (24). Here, recipients subjected to cGVHD conditions were transplanted with either Fli1fl/wt, Fli1fl/flCre+, or Fli1fl/flCre- splenocytes and WT marrow had significantly increased frequencies of CD4+CD8+ thymocytes and a lower cGVHD clinical score compared with recipients given Fli1fl/flCre+ splenocytes and WT marrow, yet recipients that received Fli1fl/flCre+ splenocytes and WT marrow grafts still had the lowest cGVHD clinical scores (Supplemental Figure 2A). Comparison of the matched splenocyte and marrow graft with the chimeric graft results suggested that T cell progenitors from Fli1fl/flCre+ marrow had a reduced ability to undergo normal CD4+CD8+ thymic reconstitution after BMT, as reflected by worse cGVHD clinical scores, whereas the recipients of Fli1fl/flCre+ grafts had improved thymic reconstitution, as reflected by low cGVHD clinical scores (Supplemental Figure 2, B and C). These data indicate that, while heterozygous Fli1 mutation did not impact thymus development, homozygous loss of this transcription factor (Fli1fl/fl) led to a significant reduction in normal thymic T cell frequencies, in agreement with previous observations that homozygous, but not heterozygous, ablation of Fli-1 impaired double-positive thymocytes (26). Thus, while a reduction of Fli-1 activity on mature T cells may be beneficial in reducing cGVHD, at least some Fli-1 activity may contribute to the conversion of T cell progenitors into CD4+CD8+ thymocytes after allo-BMT.

Fli-1 dynamically inhibits Tregs and promotes T cell IFN-γ, IL-17A, and T follicular helper–like responses in vivo. To further understand how Fli-1 regulates T cells to control GVHD disease development, we examined different types of T cell subsets and T cell phenotypes within a secondary lymphoid organ (spleen) and in peripheral lymph nodes (pLNs) of mice at late time points after BMT. Mice that received matched Fli1fl/flCre+ splenocyte and marrow grafts had enhanced donor-derived splenic CD4+ T cell reconstitution compared with mice given Fli1fl/flCre+ grafts. Only recipients of Fli1fl/flCre+ grafts had significantly higher B cell reconstitution (Figure 2A). We consistently found lower frequencies of donor-derived splenic PD-1<sup>-</sup>CXCR5<sup>-</sup>–expressing CD4+ T cells, commonly referred to as T follicular helper (Tfh) cells, in
recipients of either Fli1\textsuperscript{WT} or Fli1\textsuperscript{Cre}\textsuperscript{+} grafts compared with frequencies in recipients of Fli1\textsuperscript{WT} grafts, although only the difference between Fli1\textsuperscript{WT} and Fli1\textsuperscript{Cre}\textsuperscript{+} recipients was statistically significant. We also observed a similar phenomenon of reduced programmed cell death 1 (PD-1) expression on CD8\textsuperscript{+} T cells (Figure 2, B and C). These data suggest that Fli-1 may contribute to the differentiation of Tfh cells and CD8\textsuperscript{+} T cell activation. Within pLNs, we found significant reductions in the frequencies of donor-derived CD4\textsuperscript{+} T cells that produced IFN-\gamma in mice that received Fli1\textsuperscript{WT} or Fli1\textsuperscript{Cre}\textsuperscript{+} grafts, but only Fli1\textsuperscript{WT}Cre\textsuperscript{+} T cells had significantly reduced frequencies of CD4\textsuperscript{+}IL-17A\textsuperscript{+} T cells (Figure 2D). Frequencies of donor-derived CD4\textsuperscript{+}FoxP3\textsuperscript{+} Tregs were also increased in the recipients of Fli1\textsuperscript{WT}Cre\textsuperscript{+} or Fli1\textsuperscript{Cre}\textsuperscript{+} grafts, but only significantly in the Fli1\textsuperscript{WT} group compared with the Fli1\textsuperscript{Cre}\textsuperscript{+} group (Figure 2D).

Using the chimeric model that donor peripheral T cells can be distinguished with BM-derived T cells from WT Ly5.1\textsuperscript{+} congenic donors, we found that, compared with the Fli1\textsuperscript{WT} group, the marrow-derived cells in the recipients of Fli1\textsuperscript{WT}Cre\textsuperscript{+} grafts also had a significant reduction in the PD-1\textsuperscript{+}CXCR5\textsuperscript{+} Tfh cell-like population, along with a significant trend toward increased PD-1\textsuperscript{+}CXCR5\textsuperscript{+} cells that coexpressed CD4\textsuperscript{+}FoxP3\textsuperscript{+}, also known as T follicular regulatory-like (Tfr-like) cells (Supplemental Figure 2D). In both spleen and LNs, the recipients of Fli1\textsuperscript{WT}Cre\textsuperscript{+} T cells and WT marrow had significantly reduced frequencies of CD4\textsuperscript{+}IL-17A\textsuperscript{+} T cells compared with recipients of Fli1\textsuperscript{WT} grafts. Frequencies of CD4\textsuperscript{+}IFN-\gamma\textsuperscript{+} T cells were also reduced in the spleens and LNs of mice that received Fli1\textsuperscript{Cre}\textsuperscript{+} grafts, although the reductions were restricted to marrow-derived T cells. The recipients of Fli1\textsuperscript{WT}Cre\textsuperscript{+} grafts showed a significant trend toward increased CD4\textsuperscript{+}FoxP3\textsuperscript{+}Tregs within the marrow-derived compartment of the pLNs compared with the Fli1\textsuperscript{WT} group (Supplemental Figure 2, E–G). Taken together, these findings demonstrate that Fli-1 played an important and dynamic role in regulating the presence of pathogenic CD4\textsuperscript{+}IFN-\gamma\textsuperscript{+}Tfh, CD4\textsuperscript{+}IL-17A\textsuperscript{+}Th17, PD-1\textsuperscript{+}CXCR5\textsuperscript{+} Tfh, and protective CD4\textsuperscript{+}FoxP3\textsuperscript{+} Treg subsets in lymphoid organs of the recipient mice with cGVHD.

Fli-1 inhibits antigen-specific induced Treg function while promoting IL-2 secretion and Th17 differentiation in vitro. Little is known about the potential roles of Fli-1 in normal primary T cell biology, thus, we decided to create a T cell receptor-transgenic (TCR-Tg) mouse strain paired with our Fli1\textsuperscript{WT}CD4\textsuperscript{Cre}\textsuperscript{+} strain to study the role of this TF in antigen-specific T cell responses. CD4\textsuperscript{+} T cells from these TCR-Tg mice are only able to respond to HY-peptide (27). To study the effects of Fli-1 on the antigen-specific T cell response, we polarized CD4\textsuperscript{+} T cells with HY-peptide and different cytokine cocktails to induce Th1, Th17, or induced Treg (iTreg) differentiation. Strikingly, we found that both Fli1\textsuperscript{WT}Cre\textsuperscript{+} and Fli1\textsuperscript{Cre}\textsuperscript{+} TCR-Tg cells had enhanced iTreg (CD25\textsuperscript{+}FoxP3\textsuperscript{+}) differentiation and expression of iTreg functional molecules (CD25, CD39, CD73, and NRP-1) compared with TCRtg Fli1\textsuperscript{WT} iTregs (Supplemental Figure 3, A and B). Further, both Fli1\textsuperscript{WT}Cre\textsuperscript{+} and Fli1\textsuperscript{Cre}\textsuperscript{+} TCR-Tg cells had a significant reduction in IL-17A production compared with Fli1\textsuperscript{WT} cells under Th17-polarizing conditions (Supplemental Figure 3, C and D).
results suggest that Fli-1 contributed to the enhancement of Th17 polarization, while suppressing iTreg differentiation.

*Fli-1 regulates T cell pathogenicity in aGVHD. CD4+IFN-γ+ and/or IL-17A+ T cells play critical roles in aGVHD pathogenesis, which prompted us to determine whether Fli-1 can also contribute to aGVHD development. First, we examined early T cell activation the impact of Fli-1 on T cell growth and survival, we tested the abundance of IL-2 cytokines secreted into culture media from Th17- and Th1-polarizing cultures. We found that culture supernatants from both Fli1WT Cre+ and Fli1fl/fl Cre+ cultures had significantly reduced levels of IL-2, suggesting that Fli-1 regulated antigen-specific T cell IL-2 production (Supplemental Figure 3, E and F). Together, these

The impact of Fli-1 on T cell growth and survival, we tested the abundance of IL-2 cytokines secreted into culture media from Th17- and Th1-polarizing cultures. We found that culture supernatants from both Fli1WT Cre+ and Fli1fl/fl Cre+ cultures had significantly reduced levels of IL-2, suggesting that Fli-1 regulated antigen-specific T cell IL-2 production (Supplemental Figure 3, E and F). Together, these
and proliferation using an in vivo mixed lymphocyte reaction (MLR) model and found that donor Fli1fl/flCre+ CD4+ cells produced significantly lower levels of IFN-γ compared with both Fli1WT/WT and Fli1fl/flCre+ CD4+ cells (Figure 3A). We used the donor splenocyte and BM chimera strategy described above to determine the role of Fli-1 in aGVHD. Consistently, we found that recipients of either Fli1WT/WTCre+ or Fli1fl/flCre+ grafts had significantly increased survival rates and reduced aGVHD clinical scores compared with recipients of Fli1WT/WT grafts (Figure 3, B and C). Among all 3 experimental groups, the recipients of Fli1WT/WTCre+ grafts had the lowest aGVHD clinical scores and pathological damage in the liver, small intestine, and colon (Figure 3, D and F). Consistently, we found that T cells derived from Fli1WT/WT Cre+ donor grafts produced lower intracellular levels of IFN-γ in T cells than did Fli1WT/WT and Fli1fl/fl Cre+ donor T cells in recipient mesenteric LNs (mLNs) (Figure 3, E and G), a lymphoid organ that can closely reflect gut T cell migration and activation (28).

Indeed, alloreactive T cells are highly implicated in causing or exacerbating gut damage during GVHD (29). To extend our study beyond alloimmune responses, we used the classical syngeneic T cell transfer model of colitis to determine whether Fli-1 contributes to T cell-mediated gut damage. In support of our aGVHD findings, we observed that both Fli1WT/WTCre+ and Fli1fl/flCre+ naive CD4+ T cells had a reduced ability to induce colitis, in which Fli1WT/WTCre+ CD4+ T cells showed the least pathogenicity in colitis development (Supplemental Figure 4A). In addition, mice given Fli1WT/WTCre+ or Fli1fl/flCre+ CD4+ T cells had reduced pathological damage in the colon compared with mice given Fli1WT/WT T cells (Supplemental Figure 4, B and C). Cumulatively, these data suggest that Fli-1 dynamically contributes to IFN-γ–producing T cells during aGVHD development and that Fli-1 may be an important regulator of T cell pathogenicity in gut damage.

Fli-1 contributes to the regulation of genes involved in Treg and effector T cell development and function. To expand beyond the few target genes already known to be either positively or negatively regulated by Fli-1, we isolated purified T cells from the spleens of aGVHD mice transplanted with either Fli1WT/WT, Fli1fl/flCre+, or Fli1fl/flCre- grafts and performed next-generation RNA-Seq during the cells’ peak expansion phase (day 14 after BMT). Consistently, we found that reduced Fli-1 activity was associated with a significant reduction in aGVHD clinical scores (Supplemental Figure 5A). RNA-Seq revealed multiple significantly downregulated and upregulated genes among each of the 3 genotypes tested. When comparing Fli1WT/WTCre+ and Fli1fl/flCre+ T cells, the most significantly upregulated genes were associated with antiinflammatory properties (e.g., Foxp3, Dnase1L3, Lgals3 [galectin-3]) (30, 31), and the downregulated genes were associated with proinflammatory pathways (e.g., Egr1, Crtam, Gpr18) (32–34) (Supplemental Figure 5B). The most significantly upregulated genes in Fli1WT/WTCre+ T cells compared with WT T cells were associated with antiinflammatory properties (e.g., Foxp3, Zfp36l2, Tsc22d3 [aka GILZ]) (35), whereas the most significantly downregulated genes were related to effector T cell differentiation and function (e.g., Ifng, I2l1, Sema7a) (Supplemental Figure 5C), suggesting together with our other in vitro and in vivo data that Fli-1 may be playing an important role in Treg development, while also being able to mediate pathogenic effector T cells. Comparing Fli1fl/flCre+ T cells with WT T cells revealed a mixed proinflammatory and antiinflammatory phenotype, in which some of the most significantly upregulated genes included Ccr6, Pdcd1, Eomes, and the most downregulated genes included Tgfbi, Il6r, C1qa, C1qc, and Gzma (Supplemental Figure 5D). Genes that are involved in Treg differentiation and function, including Foxp3, Cd36 (36), Tgfbi, Il10ra (37), and Ippn5d (aka SHIP-1) were confirmed with qRT-PCR (Supplemental Figure 5E). Genes related to effector T cell structure and function — Ifng, I2l1, Fas, Gpr18 (34), and Sema7a (38) — were also confirmed via qRT-PCR (Supplemental Figure 5F).

On the basis of these RNA-Seq expression data, upstream regulator analysis via Ingenuity Pathway Analysis (IPA) also predicted significant differences in SIRT-1, NR4A1 (aka NUR77), IRF7, BCL6, TP53, and TCF7L2 pathways between Fli1WT/WTCre+ and Fli1fl/flCre+ T cells, as well as between Fli1WT/WT T cells (Supplemental Figure 6), suggesting that these pathways could be the candidates underlying the significant differences observed in gene expression among the tested genotypes.

Single-cell RNA-Seq analysis revealed discriminatory gene regulation in CD4+ versus CD8+ T cells by Fli-1. To further understand how Fli-1 regulates T cell gene transcription and heterogeneity, we performed single-cell RNA-Seq (scRNA-Seq) analysis of donor T cells, including Fli1WT/WTCre+ (Fli1WT), Fli1fl/flCre+ (Fli1fl/fl), and Fli1fl/flCre- (Fli1fl/fl) T cells, isolated from recipient mouse spleens. An unbiased integrative analysis across all 3 genotypes after regression for potential artifacts using the Seurat platform resulted in 6,501 cells grouped into 9 subpopulations, in which we recognized cluster 3 for CD4+ T cells and clusters 1 and 6 for CD8+ T cells (Supplemental Figure 7A). The CD4+ and CD8+ T cells were further clustered on the basis of differential expression of genes and visualized using uniform manifold approximation and projection (UMAP) (Figure 4A and Supplemental Figure 7B). Clustering analysis revealed 3 major subpopulations defined according to the most salient identified cell markers: early activated, effector, and memory-like in both CD4+ and CD8+ T cells (Figure 4B and Supplemental Figure 7C).

In CD4+ T cells, early activated cells were identified by the expression of the TFs Gata3, Sibt1, and Epa1; the activation markers Ptpn13, Cd69, Cd44, Il1rl1, and Klrg1; and the negative regulators Rasgrp1, Cd200r1, Soc2, and Ahrl (Figure 4, B and C) (39–41). Activation markers, including Maf, Id2, Cxcr6, Csfl, were coexpressed by early activated and effector T cells. Effector T cells were further defined by the expression of the chemokines Ccl5, Ccl3, and Ccl4; the surface molecules Nkg7, Cd2, Il2rb, Tnfrsf9, and Slamf1; and the cytokines Ifng, Gzmrb, and Il21. Memory-like T cells had the highest expression of the TFs Tcf7, Bcl2, Klf2, and Id3 and of the immune receptors Slamf6 and Slpr1 (42, 43). Consistently, the Monocle algorithm predicted a differentiation trajectory with 1 major branch point, in which early activated CD4+ T cells could form both effector and memory-like cells, further confirming the lineage relationship among 3 subsets (Figure 4D).

Loss of Fli-1 did not alter the subset distribution or the number of CD4+ T cell clusters, but rather increased the frequencies of memory-like cells relative to early activated cells in Fli1fl/fl T cells compared with Fli1WT or Fli1fl/fl CD4+ T cells (Figure 4E). Interestingly, cell-cycle analysis revealed an increased frequency of G2M- and S-phase cells in the Fli1fl/fl CD4+ T cells (Figure 4E). We conducted a gene set enrichment analysis (GSEA) to examine the potential of early activated T cells to differentiate into Th1, Th2, Th17, or Treg subsets (Figure 4F). When compared with WT controls, both Fli1fl/fl and Fli1fl/fl CD4+ T cells exhibited decreased Th1
Figure 3. Fli-1 regulates T cell pathogenicity in aGVHD. Purified T cells from spleens and LN of FlI1 WT, FlI1 Het, and FlI1 KO mice were CFSE labeled and infused into lethally irradiated BALB/c mice per mouse. Day-4 representative flow cytometry plots and cumulative frequencies of proliferated (CFSE-) donor CD4+ cells producing IFN-γ (A) (FlI1 WT, n = 6; FlI1 Het, n = 6; FlI1 KO, n = 6). Lethally irradiated BALB/c mice were transplanted with 5 × 10^5 TCD-BM from CD45.1 B6 donors supplemented or not with 0.5 × 10^6 purified total T cells from spleens and LN of FlI1 WT, FlI1 Het, and FlI1 KO donors. aGVHD representative survival rates (B) and representative aGVH clinical scores (C) (BM only, n = 7; FlI1 WT, n = 17; FlI1 Het, n = 15; FlI1 KO, n = 15). On day 14 after BMT, the indicated tissues sections were H&E stained for pathologic scoring (D), mLNs were analyzed for donor T cell populations producing IFN-γ, IL-17A, or GM-CSF. Representative flow cytometry plots display IFN-γ-producing T cells in mLNs (E), and cumulative pathology scores are shown (F) (FlI1 WT, n = 15; FlI1 Het, n = 12; FlI1 KO, n = 9). Frequencies of each indicated donor T cell population in mLNs (G) (FlI1 WT, n = 5; FlI1 Het, n = 3; FlI1 KO, n = 2). Data in A–F represent 2–3 independent experiments. Data in G were collected from 1 set of mice belonging to 3 independent experiments. Significance was determined using mixed-model tests for clinical scores, a log-rank test for survival data, and 1-way ANOVA for all other data. *P < 0.05 and ***P < 0.001.

and Th17 gene module scores, whereas FlI1KO CD8+ T cells shad the lowest module scores (Figure 4F). FlI1Het and FlI1KO CD4+ T cells had higher Treg gene module scores, and FlI1KO T cells also had an increased Th2 module score. Lower glycolysis but higher oxidative phosphorylation (OXPHOS) gene module scores were obtained in FlI1Het and FlI1KO CD4+ T cells, suggesting that FlI-1 may regulate gene pathways related to cellular metabolism (Figure 4G). In addition, compared with FlI1WT controls, FlI1KO CD4+ T cells showed downregulation of TCR pathway genes, including Cd3g, Cd3d, Lck, Cd247, Zap70, and Itk, and lower expression of TCR-induced genes, including Nkjb1, Baf1, Junf, Af4j, and Jaks (43–45). In contrast, FlI1Het CD4+ T cells showed upregulation of genes such as Zfp36l2, Lgals3, Il7r, Lax1, Ifng, and Cd226, which are involved in negative regulation of the immune effector process and among which Zfp36l2 and Lgals3 were also elevated in FlI1Het T cells in our bulk RNA-Seq data (Figure 4, H and I, and Supplemental Figure 5C) (46–49). In contrast to FlI1Het cells, fewer differentially expressed genes (DEGs) were observed when comparing FlI1KO with WT CD4+ T cells, among which Ifng was downregulated, while Zfp36l2 and Il7r were upregulated in. We observed little difference in the expression of TCR downstream TF genes, with the exception of Baff, which was downregulated in FlI1KO CD4+ T cells (Figure 4, H and I). In summary, FlI-1 deficiency in CD4+ T cells modulates gene transcription involved in T cell differentiation and metabolism. FlI-1 heterozygous deficiency in CD4+ T cells modified the composition of early activated versus memory-like cells and showed additional impact on the transcription of TCR pathway and TCR downstream TF genes.

On the other hand, 3 clusters in CD8+ T cells were identified as early activated (Ltb, Cxcr6, Id2, Ly6a, Rhp1, Plac8, Emb, Cxcr3, and Cd69), effector (Gzma, Gemb, Ifng, Prf1, Ccl3, Ccl4, Pdcd1, Lsg3, Haver2, and Eom3), and memory-like (Klf2, Vim, Sl00a4, Lgals1, Slpr1, and Ly6c2) (Supplemental Figure 7, C and D) (50). The differentiation trajectory showing that early activated CD8+ T cells could form both effector and memory cells further confirmed the cell type (Supplemental Figure 7E). FlI1Het CD8+ T cells had a similar cluster composition, whereas FlI1KO CD8+ T cells likely had decreased effector but increased memory-like cells compared with FlI1WT controls (Supplemental Figure 7F). Similar to observations in CD4+ T cells, increased frequencies of G_M-phase cells were also observed in FlI1KO CD8+ T cells (Supplemental Figure 7F). Both FlI1Het and FlI1KO CD8+ T cells had increased activation but decreased exhaustion gene module scores (Supplemental Figure 7G). FlI1Het CD8+ T cells had increased effector but reduced memory pathway gene enrichment compared with FlI1WT control CD8+ T cells, whereas FlI1KO CD8+ T cells showed an opposite trend. Consistent with the observation in CD4+ T cells, both FlI1Het and FlI1KO CD8+ T cells had increased OXPHOS, and FlI1KO CD8+ T cells showed reduced enrichment of glycolysis genes (Supplemental Figure 7H). FlI-1 deficiency in CD8+ T cells had little effect on TCR pathway and TCR downstream TF genes, and even increased the expression of Jak1 (Supplemental Figure 7I). The expression of Runx3, a TF critical for cytotoxic T lymphocyte (CTL) program initiation and memory formation (51, 52), was increased in FlI1KO CD8+, but not CD4+, T cells (Supplemental Figure 7 and Figure 4I). Thus, Fl-1 deficiency in CD8+ T cells increased gene transcription for CD8+ T cell activation, function, and OXPHOS metabolism, and Fl-1 may regulate the CD4+ versus the CD8+ T cell response differentially at the transcriptional level.

CPT, ETO, and TPT target FlI-1 and ameliorate cGVHD. To determine whether inhibiting Fl-1 could be a potential translational strategy for targeting aberrant T cell activation and GVHD, we used a currently available pharmacological agent, CPT, which has been shown previously to potently inhibit FlI-1 (15). We confirmed that CPT could reduce FlI-1 protein expression in the murine T cell leukemia line EL4 (Supplemental Figure 8A), which was associated with reduced cell growth and increased apoptosis (Supplemental Figure 8, B and C). To determine the extent of specificity of low-dose CPT for FlI-1 versus topoisomerase I inhibition, we performed a topoisomerase I enzymatic activity assay and found that low-dose CPT did not significantly hinder the ability of topoisomerase I to relax supercoiled DNA—the primary function of this enzyme—even after a 48-hour incubation of activated T cells with low-dose CPT (Supplemental Figure 8D). We then tested the impact of low-dose CPT on primary murine polyclonally activated T cells in vitro and found that CPT reduced FlI-1 expression (Supplemental Figure 1D) and T cell proliferation but preserved IFN-γ production (Supplemental Figure 8, E and F). CPT treatment of purified polyclonally stimulated WT T cells or FlI1KOCre+ T cells revealed that a low concentration of CPT had a major effect on WT T cells via decreased IFN-γ and Ki-67+ frequencies, and there were modest, nonspecific effects of CPT against FlI-1 when cultured with FlI1KOCre+ T cells (Supplemental Figure 9A). In addition, CPT treatment suppressed activated effector T cells, while sparing Tregs when stimulated with allogeneic antigen-presenting cells (APCs) in vitro (Supplemental Figure 9, B and C).

We next investigated whether this strategy would be beneficial for cGVHD prevention or treatment. CPT at low doses was able to effectively prevent cGVHD development in mice, as reflected by clinical manifestations and pathological damage in the GVHD target organs skin and small intestine (Figure 5, A–E). At a late phase of cGVHD, we found that the recipient mice treated with CPT had significantly increased numbers of CD4+ CD8+ thymocytes and significantly reduced CD4+ Tfh and CD8+ Th-like cells (Figure 5, F and G).
Figure 4. Fli-1 regulates gene transcription involved in the differentiation and function of CD4+ T cells. (A) Integrated UMAP showing 3 major CD4+ T cell clusters among donor T cells isolated from the spleens of BALB/c recipient mice that were transplanted with BM (Rag1−/−) and T cells from Fli1fl/flCre (Fl1+) and Fli1fl/flCre (Fl1−) donor mice on day 14. (B) Expression of cell-defining features across all cell types. Color intensity is proportional to the average gene expression in the indicated cell clusters. The size of the circles is proportional to the percentage of cells expressing the indicated genes. (C) mRNA expression of the indicated genes projected onto the UMAP in 3 cell subpopulations. (D) Single-cell trajectory of total CD4+ T cell subsets based on pseudotime (left) and cell type (right). (E) Integrated UMAP shows Fl1+/+, Fl1−/−, and Fl1−/+ CD4+ T cell clusters separately. Histogram shows the frequency of each cell cluster (left) and the frequency of cells in each cell-cycle phase (right) in Fl1+/+, Fl1−/−, and Fl1−/+ CD4+ T cells. (F) Dot plot shows Th1, Th2, Th17, and Treg gene module scores in early activated cells. (G) Violin plots indicate glycolysis and OXPHOS gene module scores for CD4+ T cells. (H) Volcano plots present the most DEGs between Fl1+/+ versus Fl1−/− (top) and Fl1−/+ versus Fl1−/− (bottom) CD4+ T cells. (I) Violin plots indicate the expression of the indicated genes in CD4+ T cells. Significance was determined by 1-way ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001.

In addition to cGVHD prevention, delayed CPT administration was able to effectively alleviate the severity of established cGVHD (Figure 5H). To extend our finding, we tested the efficacy of CPT using a classic model of cGVHD, in which cutaneous fibrosis develops after MHC-matched BMT (53). CPT administration starting on the day of BMT substantially alleviated cGVHD severity (Supplemental Figure 10, A–C), consistent with improved thymic CD4+CD8+ and splenic B cell reconstitution in these recipients (Supplemental Figure 10, D and E). Although CPT treatment increased CD8+ T cell production of IFN-γ, it attenuated CD4+ T cell pathogenicity, as reflected by more Foxp3-expressing, but fewer IFN-γ-producing, cells in the CD4+ population (Supplemental Figure 10F).

To determine whether another drug that could also target Fli-1 could reduce cGVHD, we tested an alternative, more clinically relevant drug (ETO) as a potential translational strategy. We found that ETO inhibited Fli-1 expression in both Jurkat cells and polyclonally activated human PBMCs compared with vehicle controls (Supplemental Figure 11), in agreement with a previous study that identified ETO as a Fli-1 inhibitor in murine erythroleukemia cells (15). When used as a prophylactic strategy in a cGVHD mouse model, ETO treatment was able to dramatically reduce cGVHD severity (Figure 6A) and was associated with significant increases in thymic CD4+CD8+ and splenic B220+ and CD4+FoxP3+ cell populations 60 days after BMT (Figure 6, B and C). We also observed a significant reduction in CD4+IFN-γ+, CD8+IFN-γ+, and CD4+IL-17A+ T cell frequencies in pLNs (Figure 6D). TPT, another drug with previously demonstrated Fli-1–inhibitory activity (16), was also able to reduce Fli-1 expression in human Jurkat cells (Supplemental Figure 12A) and attenuate cGVHD (Figure 6E), including improved thymic and B cell reconstitution, and reduced IL-17A in CD4+ T cells (Figure 6, F–H). Further, TPT reduced the frequencies of Th1-like, IFN-γ+, IL-2+, and IL-17A+ CD4+ T cells in the spleen, and reduced the frequencies of donor splenocyte–derived CD4+ and CD8+ T cells in pLNs (Supplemental Figure 12, B–F). Overall, these results suggest that targeting Fli-1 using low-dose CPT, TPT, or ETO is effective in preventing and/or reversing cGVHD, and this effectiveness can be attributed, at least in part, to Fli-1 inhibition on lymphocytes, since these drugs have established Fli-1–inhibitory activity, as shown in this study and others (15, 16).

Inhibition of Fli-1 prevents aGVHD and preserves the GVL effect. To determine the effect of low-dose CPT on the GVL effect, we used a haploidentical murine model of aGVHD supplemented with an aggressive P815 mastocytoma. In this model, mice that received T cell–depleted BM (TCD-BM) without mature T cells succumbed rapidly to P815–mediated mortality, whereas mice that were given mature T cells from allo-BMT were protected against P815 outgrowth (Figure 7A). Here, allo-BMT recipients given TCD-BM, P815, mature T cells, and vehicle developed moderate-to-severe aGVHD leading to significant GVHD-related mortality. In contrast, recipient mice under the same conditions but treated with low-dose CPT had significantly better survival (Figure 7A), reduced aGVHD clinical scores (Figure 7B), and improved thymic CD4+CD8+ reconstitution and higher donor-derived splenic B220+ B cell reconstitution compared with vehicle-treated mice (Figure 7, C and D). Importantly, while mice given TCD-BM plus P815 had rapid P815 outgrowth (100% mortality by day 14), low-dose CPT-treated mice that received mature allogeneic T cells had complete protection against P815 relapse (0 of 13 by day 80), similar to their vehicle-treated counterparts. To examine the direct effects of low-dose CPT against P815 itself, we also treated mice given TCD-BM and P815 with CPT without mature T cells, which resulted in early tumor protection, but ultimately did not directly prevent tumor relapse (100% mortality by day 30) (Figure 7, A and E). P815 cells expressed a low amount of Fli-1 compared with Jurkat cells (Figure 7F). Consistently, recipient mice administered CPT showed an intact GVL response against B cell lymphoma (A20) after allo-BMT, along with significantly reduced GVHD clinical scores (Supplemental Figure 13).

Cyclosporine is a classic immunosuppressive drug for GVHD prophylaxis. Post-transplantation cyclophosphamide (PTCy) or bendamustine has been shown to be effective in controlling GVHD development (54). We thus attempted to compare Fl1 inhibitors with these “standard” treatments. We observed that short-term treatment with CPT (4 doses) or ETO (2 doses) early after BMT effectively attenuated aGVHD severity (Supplemental Figure 14, A–C). The recipient mice administrated CPT for 2 weeks showed the most favorable outcomes, reflected by the best survival rates, the lowest clinical scores, and no leukemia relapse (Supplemental Figure 14, D–G). Importantly, using the same treatment schedule as for PTCy, 2 doses of CPT or ETO administered on days 3 and 4 were sufficient to prevent GVHD and leukemia relapse. In contrast, some of the recipients treated with bendamustine succumbed to GVHD, while some of the mice treated with PTCy or cyclosporine experienced leukemia relapse. Furthermore, long- or short-term administration of CPT, ETO, or TPT did not show toxicity to hematopoietic stem cells (HSCs), as reflected by similar numbers of donor-derived HSCs in the recipients’ BM (Supplemental Figure 15, A–C). Inhibition of Fli-1 with CPT, ETO or TPT did not delay myeloid cell reconstitution, or even improved CD11b+ cell reconstitution in PB and spleens from mice treated with CPT or ETO for 2 weeks (Supplemental Figure 15, D and E).

We then tested the ability of T cells with heterozygous or homozygous Fli1 deficiency to mediate the GVL effect against P815 and...
found that mice given Fli1\(^{fl/WT}\)Cre\(^{+}\) T cells were able to survive long term (Figure 7G), while also maintaining a lower aGVHD clinical score (Figure 7H) and a strong GVL effect against P815 (Figure 7I) compared with mice that received WT T cells. Consistently, the recipients of Fli1\(^{fl/WT}\)Cre\(^{+}\) T cells also showed improved clinical manifestations and maintained GVL responses. A recent study demonstrated that Fli-1 represses effector CD8\(^{+}\) T cell responses during anti-infection and antitumor responses \(14\). We further studied the role of Fli-1 in regulating CD8\(^{+}\) T cell responses during GVL activity after allo-BMT. Compared with WT control CD8\(^{+}\) T cells, Fli1-deficient CD8\(^{+}\) T cells in both the spleen and liver had an enhanced ability to become memory precursor effector cells (KLRG1\(^{CD127^{+}}\)) and expressed lower Lag3 in the liver. With the exception of IFN-\(\gamma\), both control CD8\(^{+}\) T cells and Fli1-deficient CD8\(^{+}\) T cells had comparable expression levels of granzyme B, TNF-\(\alpha\), CD107a, Fas-L, PD-1, and CXCR3 (Supplemental Figure 16). Taken together, these results indicate that T cells with either heterozygous or homozygous Fli1 deficiency maintained their GVL activity. Additionally, we found that targeting Fli-1 using low-dose CPT or ETO was an effective strategy to reduce aGVHD severity and lethality after allo-BMT, while preserving the ability of alloreactive T cells to prevent leukemia relapse.

CPT inhibits Fli-1 on human T cells and reduces GVHD in a xenograft model. To further increase the clinical relevance of our study,
we tested CPT in human cells and found that CPT at a very low dose was able to potently inhibit Fli-1 protein levels in a human transformed T cell line (Jurkat) (Supplemental Figure 17A). Jurkat cells had reduced growth and markedly induction of apoptosis via CPT by culture day 3 compared with vehicle treatment (Supplemental Figure 17, B and C). CPT was also a potent inhibitor of Fli-1 in polyclonally stimulated human PBMCs in vitro (Figure 8A). To further confirm Fli-1 protein expression specifically in
were observed, although their ability to produce IFN-γ was not reduced on a per-cell basis (Figure 9, D and E). We then confirmed that CPT also acts as a Fli-1 inhibitor in vivo, as cells extracted from splenocytes of mice treated with CPT showed an obvious reduction in Fli-1 protein levels (Figure 9F). To study how CPT affects human Tregs, we performed a separate xenograft GVHD experiment and observed improved body weight maintenance and survival of mice that received CPT treatment (Supplemental Figure 18A), but also found that the frequencies of human Tregs in CPT-treated mice were not reduced in the spleens of recipient mice and were modestly elevated in recipients’ livers compared with vehicle-treated mice (Supplemental Figure 18, B and C). In addition, CD25+CD4+ and CD25+CD8+ T cells expressed the highest levels of Fli-1, especially after activation, while Foxp3+ and CD25 Foxp3- cells maintained the lowest Fli-1 expression, which might be explained by the inhibition of Fli-1 that spared Tregs.
to posit several potential mechanisms that would explain how Fli-1 regulates the allogeneic T cell response during GVHD development.

Particularly interesting in our study was the finding that, in genotype-matched spleen and BM cGVHD transplants, T cell–specific heterozygous Fli-1 reduction led to distinctly different outcomes with regard to clinical score and T cell phenotypes when compared with both groups: mice with homozygous reduction of Fli-1 activity and WT mice. However, these disparate cGVHD clinical score outcomes between the heterozygous and homozygous groups were largely diminished when the source of donor BM was changed from $\text{Fli1}^{-/-}\text{Cre}^{+}$ to WT marrow, although several differences still remained regarding T cell phenotypes. When we switched $\text{Fli1}^{-/-}\text{Cre}^{+}$ (Supplemental Figure 18D). Consistently, we found no evident impairment of body weight maintenance or myeloid cell reconstitution after a full course of CPT treatment (Supplemental Figure 18, E–G). Cumulatively, these data indicate an ability of CPT to act as a Fli-1 inhibitor on primary human lymphocytes and that CPT can reduce human T cell proliferation as well as improve the survival of graft recipients in a xenograft model.

Discussion

The specific role of Fli-1 in primary CD4$^+$ T cells has not been studied in depth to date, especially not in allo-HCT conditions. Our findings, combined with those in the previous literature, allow us to posit several potential mechanisms that would explain how Fli-1 regulates the allogeneic T cell response during GVHD development.

Particularly interesting in our study was the finding that, in genotype-matched spleen and BM cGVHD transplants, T cell–specific heterozygous Fli-1 reduction led to distinctly different outcomes with regard to clinical score and T cell phenotypes when compared with both groups: mice with homozygous reduction of Fli-1 activity and WT mice. However, these disparate cGVHD clinical score outcomes between the heterozygous and homozygous groups were largely diminished when the source of donor BM was changed from $\text{Fli1}^{-/-}\text{Cre}^{+}$ to WT marrow, although several differences still remained regarding T cell phenotypes. When we switched $\text{Fli1}^{-/-}\text{Cre}^{+}$

Figure 8. CPT acts as a Fli-1 inhibitor on human T cells and reduces their proliferation in vitro. Total human PBMCs isolated from healthy donors were CFSE labeled and activated in vitro via soluble anti-CD3/anti-CD28 (2 μg/mL) and cocultured with DMSO (vehicle) or 15 nM CPT for 3 days. On day 3, cultures were harvested and lysed for Western blot analysis of Fli-1 protein expression, with β-actin as the loading control (A). Representative flow cytometric histograms show intracellular Fli-1 expression in CD3$^+$CD4$^+$ and CD3$^+$CD4$^+$ gated T cells treated with vehicle or 15 nM CPT (left) and representative Fli-1 MFI values (right); isotype (Iso) control (red line); secondary antibody only (blue line); vehicle-treated cells (green line); CPT-treated cells (orange line) (B). Max, maximum. Representative flow cytometric plots show proliferation (CFSE dilution) and IFN-γ cytokine production in human T cells (C). SSC-A, side scatter area. Representative frequencies of live cells in culture (left), CD4 proliferation and cytokine production (middle), and CD8 proliferation and cytokine production (right) are shown (D). Data represent 2 independent experiments, each performed in triplicate except for Western blotting, in which triplicate wells were combined into single lysates for each condition, and 2 independent blots were performed. Significance was determined using an unpaired, 2-tailed Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
BM to WT BM, we noted a substantial increase in the frequency of CD4^+CD8^+ thymocytes during cGVHD. Indeed, a previous group discovered that germline heterozygous mutation of *Fli1* resulted in normal thymus development, but homozygous mutation resulted in a significant reduction in thymocyte numbers that was attributed to defects in prethymic T cell progenitors (26). This report is consistent with our findings that a homozgygous, but not a heterozygous, reduction of Fli-1 activity on donor BM–derived T cells could impair the frequency of CD4^+CD8^+ thymic T cell repopulation after allo-BMT, suggesting that at least 1 allele of *Fli1* was required for optimal thymic reconstitution. Bulk RNA-Seq and qRT-PCR analyses suggested that, upon T cell activation with alloantigen, Fli-1 can contribute to the regulation of genes associated with activation and inflammation, as well as anti-inflammatory T cell genes that can contribute to and suppress GVHD development, respectively.

The scRNA-Seq analysis of the donor T cells isolated from the allo-BMT recipients indicated a major difference in the transcriptional regulation by Fli-1 in CD4^+ versus CD8^+ T cells and in the *Fli1* gene dose-dependent modification of transcriptional pathways in Fli<sup>1/2</sup> versus Fli<sup>1/0</sup> T cells. Fli-1 was deemed to play a distinct role in the regulation of gene expression in donor BM–derived T cells after allo-BMT.

Figure 9. CPT inhibits human Fli-1 and reduces GVHD in a xenograft model. HLA-A2^+ NSG mice were sublethally irradiated (250 cGy) and transplanted with 8 × 10^7 to 10 × 10^7 total human PBMCs from a healthy donor (HLA-A2^−) to induce human GVHD. These mice received vehicle or CPT at 0.25–0.5 mg/kg on day 0, which was then every other day until day 14 after BMT. Recipient survival rates (A) and body weights (B) were monitored up to 80 days after transplantation. Peripheral blood staining of human (Hu) CD8^+ T cells on day 14 after transplantation (C), and percentage and number of human IFN-γ–producing CD3^+CD8^+ T cells (top) and human IFN-γ–producing CD3^+CD8^+ T cells (bottom) (E). Western blot of day-15 splenic whole-cell lysates from 4 vehicle-treated and 4 CPT–treated xenografted mice using the indicated primary antibodies (F). Data in A and B represent 2 independent experiments (IRR only, n = 6; IRR + PBMCs + vehicle, n = 10; IRR + PBMCs + CPT, n = 10). Data in C–F were collected from 1 set of mice belonging to 2 independent experiments. Significance was determined using mixed-model tests for body weight, a log-rank test for survival data, and an unpaired, 2-tailed Student’s t test for all other data. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. IRR, irradiation.
role in regulating gene transcription in CD4+ versus CD8+ T cells, in that more activation and fewer exhaustion gene pathways were enriched in CD8+ Fli-1-deficient T cells, whereas fewer Th1/Th17 pathogenic pathways were enriched in CD4+ Fli-1-deficient T cells. Overall, Fli-1 deficiency increased gene enrichment in the OXPHOS pathway in both CD4+ and CD8+ T cells and substantially reduced gene enrichment in the glycolysis pathway in CD4+ T cells. We and others reported that alloreactive T cells upregulate essential metabolic pathways, in which glycolysis manifests as a major source of energy for GVHD-inducing T cells (59). On the other hand, lower levels of glycolysis and increased levels of OXPHOS are beneficial to the generation of long-lived memory T cells for persistent antitumor activity (56). Thus, metabolic modification and differential regulation of CD4+ versus CD8+ T cell responses in Fli-1 deficiency may be beneficial for maintaining GVCL activity, while attenuating GVHD pathogenicity. Consistent with the lowest pathogenicity of Fli1Het KO T cells for GVHD induction, we observed that Fli1Het CD4+ T cells had the lowest Th1/Th17 pathway enrichment and TCR pathway downstream gene expression. Further study is required to define the mechanism by which different doses of the Fli-1 gene regulate the expression and function of these TCR downstream TFs in modifying CD4+ and CD8+ T cell responses.

In a recent report, Chen et al. found that Fli-1 antagonized the differentiation of KLRG1hi Teff cells during acutely resolved infection and also chronic infection mediated by antigen-specific CD8+ T cells (14). They elegantly demonstrated that Fli-1 inhibited T-effector-like (Teff-like) cell differentiation by coordinating with Runx1 and antagonizing Runx3 function. Interestingly, in our allo-BMT models, Fli1Het CD8+ T cells showed comparable frequencies of KLRG1hiCD127+ Teff cells, but greater frequencies of KLRG1loCD127+ memory precursor cells, than did WT controls in recipient spleens and livers. Fli1Het CD8+ T cells produced less IFN-γ in recipient spleens and lower levels of Lag3 in recipient livers. Similarly, in our scRNA-Seq analysis, Fli1Het CD8+ T cells showed higher enrichment for memory genes, but lower enrichment for effector pathway genes. On the other hand, Fli1Het CD8+ T cells had more effector but less memory gene enrichment compared with WT controls, suggesting a possible gene dose effect of Fli-1 on CD8+ T cell differentiation. Consistent with this study, we found that Fli1Het CD8+ T cells expressed higher levels of Runx3, a TF critical for epigenetic modification and differentiation of CD8+ CTLs into effector memory cells and tissue-resident memory cells (51, 52). Furthermore, both Fli1Het and Fli1Ko CD8+ T cells had higher activation but lower exhaustion gene module scores than did WT controls, suggesting that Fli-1 may negatively regulate CD8+ T cell function during the allologeneic response. However, since CD4+ T cells were the predominant T cell subset that drove GVHD pathogenesis in these tested models, we interpret this to mean that the GVHD alleviation resulted from the reduced CD4+ T cell activation in allo-BMT. Under this condition, CD8+ T cells did not exhibit higher effector function and maintained greater memory programming in the absence of Fli-1.

There has still been relatively little progress in the field in the development of a Fli-1-specific inhibitor, despite its known involvement in multiple types of malignancies. Therefore, some of the only pharmacological strategies available to date that can inhibit Fli-1 are known chemotherapy drugs such as CPT, TPT, and ETO. Thus, we used each of these drugs in our study to determine whether targeting Fli-1 pharmacologically would be beneficial in allo-HCT. We observed that CPT did not obviously impair the enzymatic activity of topoisomerase I at low concentrations in activated murine T cells. It was also previously reported that CPT is significantly less effective at preventing the growth of malignant cell lines designed to overexpress Fli-1 (15). The current study further supports our recent report that low doses of CPT or TPT inhibit Fli-1 and significantly attenuate lupus nephritis without liver toxicity or myelosuppression (57). Taken together, these results suggest that CPT or TPT acts through Fli-1 inhibition as an important and currently underappreciated mechanism of action. Nonetheless, beyond targeting Fli-1, we cannot exclude other potential mechanisms by which the topoisomerase inhibitors alleviated GVHD in vivo, given that topoisomerases are involved in DNA repair, replication, and transcription during mitosis (58). Inhibition of topoisomerase by CPT, ETO, or TPT could induce apoptosis of activated T cells during the G1 to S-phase transition (59), reduce the expression of MHC-II and costimulatory molecules on APCs (60, 61), or activate the stimulator of IFN genes (STING) pathway (62) and other Fli-1-regulated inflammatory factors, such as CXCR3, IL-6, C16-deramide, GM-CSF, and mir-17-92 (4–8). All of these pathways have been shown by us and others to critically contribute to GVHD pathogenesis (9, 10–13, 63).

We used low-dose CPT and examined its effect on GVHD prevention and leukemia control. We found in subsequent studies involving allo-HCT experiments that P815 expressed low levels of Fli-1 compared with other cell lines such as the Jurkat cell line. This could potentially explain why there was an early benefit of CPT administration against P815. We observed that the GVL response against P815 or A20 was not impaired by CPT treatment. In agreement with our data showing the ability of CPT to inhibit Fli-1 and preserve the GVL effect, both Fli1HetCre+ T cells and Fli1HetCre+ T cells also had the ability to preserve the GVL effect.

In addition, ETO, as well as TPT, was able to reduce Fli-1 expression and cGVHD development through suppression of inflammatory T cell responses, while sparing Tregs in lymphoid tissues, in agreement with a previous report showing that ETO was able to selectively target activated T cells (64). While human effector T cells were reduced in both the spleen and the liver, human CD4+FoxP3+ Treg were not reduced after CPT treatment, suggesting that CPT can selectively target effector T cells without impairing Tregs. These findings are consistent with our in vitro data showing that inhibition of Fli-1 enhanced murine iTreg numbers and functional molecules. These effects of CPT, TPT, and ETO may be due, at least in part, to a reduction of Fli-1 activity (15, 16). Interestingly, ETO used in the clinic as a myeloablative conditioning regimen has compared favorably with other agents such as cyclophosphamide for the ability to reduce leukemia relapse and GVHD severity (65, 66). Thus, it is worth exploring in future studies whether currently utilized chemotherapeutic agents such as TPT, ETO, and other chemically related drugs (e.g., irinotecan) could be repurposed as strategies to reduce Fli-1 activity and prevent or treat GVHD in the clinical setting. Furthermore, more highly specific Fli-1 inhibitors have recently been identified that will promote the targeting of Fli-1 as an interventional strategy in clinical applications (67).

In preclinical studies, PTCy was found to be effective in preventing GVHD that was largely attributed to selective elimination of alloreactive T cells, functional impairment of alloreactive T cells, and...
preferential recovery of CD4+ Tregs (68, 69). High-dose cyclophosphamide (50 mg/kg) given on day 3 or days 3 and 4 after transplantation was associated with a low incidence of aGVHD but extensive cGVHD in patients following nonmyeloablative HLA-haploidentical HCT. However, malignant relapse was a major reason for treatment failure in these patients with high-risk hematologic malignancies and was possibly caused by cyclophosphamide-mediated deletion of tumor-specific CD8+ T cells (54, 70). In the setting of myeloablative conditioning regimens, although relapse rates were reduced, increased GVHD and nonrelapse mortality were observed (71, 72). An alternative strategy to separate T cell GVH and GVL responses is highly warranted. We directly compared the outcomes of CPT, ETO, and TPT versus PTCy treatment using a haploidentical model of GVHD and found that 2 weeks of CPT treatment showed the best outcomes, free of GVHD and leukemia relapse (Supplemental Figure 14, D–G). Two doses of CPT or ETO early after BMT were as effective as PTCy in preventing GVHD and had a less negative impact on the GVL effect and were thus more effective in controlling leukemia relapse. Therefore, targeting Fli-1 using a pharmacological strategy could be potentially beneficial in the allo-HCT setting by (a) targeting leukemias and lymphomas that overexpress or rely on Fli-1; and (b) targeting pathogenic alloreactive T cells that utilize Fli-1 to some extent for differentiation, survival, or cellular functions. Overall, these results suggest that strategies to reduce Fli-1 expression or transcriptional activity may be a promising area of future research for therapies that aim to reduce GVHD development without compromising the ability of T cells to mediate antileukemia activity. The identification and implementation of specific Fli-1 inhibitors will further promote the translation of our findings into clinical applications.

Methods

Experimental mice. Female and male BALB/c (H-2b), C57BL/6 (B6), Ly5.1 (H-2b, CD45.1), B6.Ly5.2 (H-2b, CD45.2), and (BALB/c x DBA2)F1 (B6D2F1, H-2b) mice were purchased from Charles River Laboratories. Rag1<−/−> and NSG mice (NOD.Cg-Prkdc<cscl2>Il2rg<tm1Wsj>Tg(HLA-A-H2-D/R2M1Dvs/SzJ; stock no. 014570) were purchased from The Jackson Laboratory. Fli1<−/−> mice on a B6 background were a gift from Xian Zhang’s group (MUSC, Charleston, South Carolina, USA) (19). T cell conditional deletion of Fli1 exon 3 and 4 was mediated by a Cre/lox system utilizing the CD4+ promoter. Homozygous Fli1 exon 3 and 4 deletion (referred to as Fli1<−/−> Cre−) was mediated via Fli1<−/−> CD4Cre<−>; heterozygous Fli1 exon 3 and 4 deletion (referred to as Fli1<+/−> Cre−) was mediated via Fli1<+/−> CD4Cre<−>; and WT controls (referred to as Fli1<+/+> Cre−) were Fli1<+/+>. CD4Cre<−>, Fli1<WT> CD4Cre<−>, or Fli1<WT> CD4Cre<−>. Fli1<−/−> CD4Cre<−> mice were also crossed with Marilyn transgenic mice, described previously (27), to generate HY-antigen–specific T cells with reduced Fli-1 activity. All mice were maintained in a specific pathogen–free facility at an American Association for Laboratory Animal Care–accredited Animal Resource Center at the MUSC and the MCW. Mice were randomly assigned to groups for all relevant experiments, and both female and male donor and recipient mice were tested in genetic and pharmacological experiments.

Experimental procedures and statistics. Allo-BMT, the GVL model, treatment with Fli-1–inhibiting drugs, and statistical analyses are described in detail in the Supplemental Methods. RNA-Seq raw data files can be found in the Sequence Read Archive (SRA) database (SAMN30526153, SAMN30526154, SAMN30526155, SAMN30526295, SAMN30526296, and SAMN30526297).

Study approval. All animal experiments were approved by the IACUC of the MUSC and the Animal Use Application (AUA) of the MCW.

Author contributions

SDS participated in experimental design, performed research, collected, analyzed, and interpreted data, performed statistical analysis, and drafted and revised the manuscript. YW participated in experimental design, performed research, collected, analyzed, and interpreted data, performed scRNA-Seq data analysis and statistical analysis, and revised the manuscript. AK analyzed scRNA-Seq data. DB, HJC, MHS, CM, BMM, and HN performed research and interpreted data. CL and KH performed histological scoring of mouse tissues. YBD participated in experimental design, interpreted data, and revised the manuscript. WC interpreted data and revised the manuscript. XZ participated in experimental design, interpreted data, revised the manuscript, and generated genetically modified Fli1-mice. XZY designed research, interpreted data, performed statistical analysis, and revised the manuscript.

Acknowledgments

The Division of Laboratory Animal Resources (DLAR) of the MUSC and the MCW provided valuable animal husbandry and care support for this study. The research presented in this article was supported in part by the Flow Cytometry and Cell Sorting Unit at both institutions. This work was supported in part by the National Center for Advancing Translational Sciences of the NIH (TL1 TR001451 and UL1 TR001450). This work was also supported in part by the Hollings Cancer Center Graduate Fellowship and the Cell Evaluation and Therapy Shared Resource, Hollings Cancer Center, MUSC (P30 CA138313). This study was supported in part by the NIH, including the R01 grants AL118305, HL140953, and CA258440 (to XZY).

Address correspondence to: Xue-Zhong Yu and Yongxia Wu, Department of Microbiology and Immunology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, USA. Phone: 414.955.8187; Email: xyuow@mcw.edu (XZY); Phone: 414.955.8148; Email: wyongxia@mcw.edu (YW).
SHIP-1 is negatively regulated by Fli-1 and its loss accelerates leukemogenesis. *Blood.* 2010;116(3):428–436.

25. Zlotoff DA, Bhandoola A. Hematopoietic progenitor migration to the adult thymus. *Ann N Y Acad Sci.* 2011;1217:122–138.

26. Melet F, et al. Generation of a new Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia. *Mol Cell Biol.* 1996;16(6):2708–2718.

27. Li J, et al. HT-specific induced regulatory T cells display high specificity and efficacy in the prevention of acute graft-versus-host disease. *J Immunol.* 2015;195(2):717–725.

28. MacPherson AJ, Smith K. Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med.* 2006;203(3):497–500.

29. Piper C, Drobyski WR. Inflammatory cytokine networks in gastrointestinal tract graft vs. host disease. *Front Immunol.* 2019;10:163.

30. Sisirak V, et al. Trafficking of transcription factor Fli-1 and topoisomerase, markers of Fli-1 inhibitory activity suppress diverse types of leukemia. *J Immunol.* 2017;46:14–22.

31. Cipolletta D, et al. PPAR γ is a major driver of the accumulation and phenotype of adipose tissue Tregs. *Nature.* 2012;486(7404):549–553.

32. Chaudhry A, et al. Interleukin-10 signaling in regulatory T cells is required for establishment of the CD8 effector T cell compartment. *Front Immunol.* 2018;9:660.

33. Ayrolde E, et al. Modulation of T cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappa B. *Blood.* 2001;98(3):743–753.

34. Suzuki K, et al. Semaphorin 7A initiates immune synapse formation by the tyrosine phosphatase PTP-BL. *Nat Commun.* 2018;9:2826.

35. Ciucci T, et al. The emergence and functional fit of Tcf1 long isoforms in CD8+ T cell responses to CD4+ T cell responses to acute viral infection. *J Immunol.* 2017;199(5):911–919.

36. Li Y, et al. Regulation of signal transduction and a delay in Friend virus-induced erythropoiesis in NZBWF1 mice and reduce the production of inflammatory mediators in human renal cells. *Arthritis Rheumatol.* 2021;73(8):1478-1488.

37. Song J, et al. Novel flavagline-like compounds with potent Fli-1 inhibition activity suppress diverse types of leukemia. *FEBS J.* 2018;285(4):4631–4645.

38. Asano Y, et al. Endothelial Fli1 deficiency impairs disease in mice by camptothecin and etoposide induce a CD95-independent apoptosis of activated peripheral lymphocytes. *Cell Death Differ.* 2000;7(2):197–206.

39. Nakahira M, et al. Regulation of signal transduction and a delay in Friend virus-induced erythropoiesis in NZBWF1 mice and reduce the production of inflammatory mediators in human renal cells. *Arthritis Rheumatol.* 2021;73(8):1478-1488.

40. Chaudhry A, et al. Interleukin-10 signaling in regulatory T cells is required for establishment of the CD8 effector T cell compartment. *Front Immunol.* 2018;9:660.

41. Ayrolde E, et al. Modulation of T cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappa B. *Blood.* 2001;98(3):743–753.

42. Cho SH, et al. Hypoxia-inducible factors in CD4+ T cells promote metabolism, switch cytokine secretion, and T cell help in humoral immunity. *Proc Natl Acad Sci U S A.* 2019;116(18):8975–8984.

43. Schober SL, et al. Expression of the transcription factor lung Krüppel-like factor is regulated by cytokines and correlates with survival of memory T cells in vitro and in vivo. *J Immunol.* 1999;263(7):3662-3667.

44. Guziklsrud JA, et al. Differential requirements for Tcf7 long isoforms in CD8+ and CD4+ T cell responses to acute viral infection. *J Immunol.* 2017;199(5):911–919.

45. Li Y, et al. Regulation of signal transduction and a delay in Friend virus-induced erythropoiesis in NZBWF1 mice and reduce the production of inflammatory mediators in human renal cells. *Arthritis Rheumatol.* 2021;73(8):1478-1488.
topoisomerase II inhibitor-induced tumor immunogenicity. J Clin Invest. 2019;129(11):4850–4862.
63. Wu Y, et al. STING negatively regulates allogeneic T cell responses by constraining antigen-presenting cell function. Cell Mol Immunol. 2021;18(3):632–643.
64. Johnson TS, et al. Etoposide selectively ablates activated T cells to control the immunoregulatory disorder hemophagocytic lymphohistiocytosis. J Immunol. 2014;192(1):84–91.
65. Czyz A, et al. Cyclophosphamide versus etoposide in combination with total body irradiation as conditioning regimen for adult patients with Ph-negative acute lymphoblastic leukemia undergoing allogeneic stem cell transplant: On behalf of the ALWP of the European Society for Blood and Marrow Transplantation. Am J Hematol. 2018;93(6):778–785.
66. Salhotra A, et al. Long-term outcomes of patients with acute myelogenous leukemia treated with myeloablative fractionated total body irradiation TBI-based conditioning with a tacrolimus- and sirolimus-based graft-versus-host disease prophylaxis regimen: 6-year follow-up from a single center. Biol Blood Marrow Transplant. 2019;26(2):292–299.
67. Liu T, et al. Identification of diterpenoid compounds that interfere with Fli-1 DNA binding to suppress leukemogenesis. Cell Death Dis. 2019;10(2):117.
68. Wachsmuth LP, et al. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. J Clin Invest. 2019;129(6):2357–2373.
69. Nunes NS, Kanakry CG. Mechanisms of graft-versus-host disease prevention by post-transplantation cyclophosphamide: an evolving understanding. Front Immunol. 2019;10:2668.
70. Munchel AT, et al. Treatment of hematological malignancies with nonmyeloablative, HLA-haploidentical bone marrow transplantation and high dose, post-transplantation cyclophosphamide. Best Pract Res Clin Haematol. 2011;24(3):359–368.
71. Ciurea SO, et al. Haploidentical transplant with posttransplant cyclophosphamide vs matched unrelated donor transplant for acute myeloid leukemia. Blood. 2015;126(8):1033–1040.
72. Solomon SR, et al. Total body irradiation-based myeloablative haploidentical stem cell transplantation is a safe and effective alternative to unrelated donor transplantation in patients without matched sibling donors. Biol Blood Marrow Transplant. 2015;21(7):1299–1307.