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JAK3 Mutations and Mitochondrial Apoptosis Resistance in T-Cell Acute Lymphoblastic Leukemia

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AUTHOR CONTRIBUTIONS

K.B. and A.G. conceived the project. K.B., N.Y., A.B., J.L., T.N.C., and M.B. designed and performed experiments and interpreted data. K.E.S. and M.D. performed statistical analyses and interpreted data. M.L.L, S.P.H. B.W., L.B.S., D.T.T. J.P.M. and A.L. aided in data collection of primary patient samples, and in analysis and interpretation of data. K.B., N.Y. and A.G. wrote the paper with input from all co-authors.

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Abstract

Resistance to mitochondrial apoptosis predicts inferior treatment outcomes in patients with diverse tumor types, including T-cell acute lymphoblastic leukemia (T-ALL). However, the genetic basis for variability in this mitochondrial apoptotic phenotype is poorly understood, preventing its rational therapeutic targeting. Using BH3 profiling and exon sequencing analysis of childhood T-ALL clinical specimens, we found that mitochondrial apoptosis resistance was most strongly associated with activating mutations of JAK3. Mutant JAK3 directly repressed apoptosis in leukemia cells, because its inhibition with mechanistically distinct pharmacologic inhibitors resulted in reversal of mitochondrial apoptotic blockade. Inhibition of JAK3 led to loss of MEK, ERK and BCL2 phosphorylation, and BH3 profiling revealed that JAK3-mutant primary T-ALL patient samples were characterized by a dependence on BCL2. Treatment of JAK3-mutant T-ALL cells with the JAK3 inhibitor tofacitinib in combination with a spectrum of conventional chemotherapeutics revealed synergy with glucocorticoids, in vitro and in vivo. These findings thus provide key insights into the molecular genetics of mitochondrial apoptosis resistance in childhood T-ALL, and a compelling rationale for a clinical trial of JAK3 inhibitors in combination with glucocorticoids for patients with JAK3-mutant T-ALL.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is a malignancy of immature T-lymphoblasts that accounts for approximately 15% of cases of acute lymphoblastic leukemia in children (1). The T-cell immunophenotype was associated with an increased risk of relapse prior to the implementation of risk-adapted therapy (2-4), but the intensification of conventional therapy has improved outcomes for these patients (5-8). Several contemporary treatment regimens now further intensify cytotoxic therapy for T-ALL patients who exhibit evidence of a poor response to initial combination chemotherapy, as assessed by minimal residual disease (MRD) monitoring (9-12). However, this further intensified therapy is associated with an increased risk of acute and long-term toxicity, which can be fatal (9, 13-16). Moreover, patients with T-ALL that proves resistant to such intensified therapy have a dismal prognosis (17-19). These findings highlight the need for more effective and less toxic treatment options for patients with T-ALL at increased risk of relapse. Such approaches could also lead to the development of less toxic therapies for patients with a more favorable prognosis, if molecularly targeted therapies can successfully replace the most toxic elements of contemporary therapy.

Mitochondrial apoptotic priming is a robust predictor of resistance to cancer chemotherapy in patients with a broad range of tumor types, including T-ALL (20-23). However, the biologic basis for the striking variability in this cellular phenotype across patients with apparently indistinguishable tumors are not well understood. We recently found that mitochondrial apoptosis resistance in T-ALL can be induced by loss-of-function mutations of the polycomb repressive complex 2 (PRC2) (20). However, there are currently no clinical drugs that can be used to target polycomb-inactivating mutations, and we still lack a molecular mechanism to explain apoptosis resistance in a substantial fraction of children

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with treatment-resistant T-ALL. Here, we report that JAK3 gain-of-function mutations induce resistance to mitochondrial apoptosis in pediatric T-ALL, and provide a rational strategy for therapeutic intervention.

METHODS

T-ALL clinical specimens were collected with informed consent and institutional review board approval in accordance with the Declaration of Helsinki from children with newly diagnosed T-ALL enrolled on Dana-Farber Cancer Institute (DFCI) Study 05-001 (24), or Children's Oncology Group (COG) AALL0434 (25) clinical trials.

Complete methods are included in the Supplementary Methods.

RESULTS

JAK3 mutations are associated with resistance to mitochondrial apoptosis in human T-ALL.

To further unravel the molecular genetics of apoptosis resistance in T-ALL, we performed fluorimeter-based BH3 profiling and targeted exome sequencing analysis on a cohort of diagnostic T-ALL clinical specimens collected from children enrolled on clinical trials DFCI 05-001 or COG AALL0434. This revealed that JAK3 mutations, which were present in 25% (n = 10 of 40) of cases analyzed by BH3 profiling analysis, were the strongest predictor of resistance to mitochondrial apoptosis identified (Figure 1a, Supplementary Table 1, Supplementary Table 2, Supplementary Table 3). There was also a weaker association of apoptosis resistance with mutations of the PTEN-PI3K-AKT and RAS-NF1 pathways when these were considered together, but not individually (Supplementary Table 3). NOTCH1 mutations were somewhat more common in JAK3-mutant than in JAK3 wild-type cases (n = 13 of 16 vs. n = 50 of 93; $P = 0.055$ by Fisher's exact test; Supplementary Figure 1, Supplementary Table 4), however NOTCH1 mutations lacked any association with apoptosis resistance (Supplementary Table 3, $P = 0.89$). We thus focused our study on JAK3 mutations. Most JAK3 mutations occurred near the kinase or pseudo kinase domains (Figure 1b) and were present at variant allele fractions consistent with heterozygous mutations in the bulk tumor population (Supplementary Table 1).

To assess whether the JAK3 mutations identified are pathogenic, we tested their ability to rescue interleukin-3 (IL-3)-dependent Ba/F3 cells from IL-3 withdrawal. Ba/F3 cells were transduced with each of the mutated alleles, wild-type JAK3 or EGFP negative controls. The transduced cells were removed from IL-3, and cell viability was assessed every two days. This revealed that the previously reported JAK3-activating mutations (M511I, V674A, L857P, V678M) (26-28) identified in this cohort were able to substitute for IL-3 signaling in these cells, while cells transduced with JAK3 wild-type or EGFP negative controls were not (Figure 1c). Furthermore, we identified 3 new pathogenic mutations (815insE, R549Q, R887C) near the kinase or pseudo kinase regions of JAK3 (Figure 1c). The association of JAK3 mutations with mitochondrial apoptosis resistance was further strengthened when we excluded mutations that were not pathogenic on the Ba/F3 IL-3 dependency assay, or that had variant allele frequencies <0.2, which suggests that a substantial fraction of blasts in

those samples were JAK3 wild-type (Figure 1d). Apoptosis resistance was associated with JAK3 variant allele fractions that approached heterozygosity in the bulk tumor population (Supplementary Figure 2a). While the presence of biallelic JAK3 mutations has been linked to further increases in STAT activation and T-cell transformation (29), there were no obvious differences in apoptosis resistance between cases with intermediate vs. strong JAK3-activating mutations, or with mono- vs. bi-allelic mutations (Supplementary Figure 2b-c).

We then asked whether JAK3 mutations predict treatment response in T-ALL cases analyzed by sequencing analysis for which clinical treatment response data were available (Supplementary Table 1). JAK3 mutations were associated with a poor response to initial induction chemotherapy, as assessed by 5% residual leukemia in the bone marrow after 4 weeks of induction chemotherapy (Figure 1e). This cutoff was selected because it was the lowest cutoff used to define induction failure on the clinical trials from which our samples were obtained (DFCI 05001), and recent data suggests that 5% minimal residual disease (MRD) or frank induction failure are similarly poor prognostic markers, at least in some treatment contexts (30). However, JAK3 mutations were not associated with an increased incidence of frank disease recurrence (Figure 1f), nor with inferior overall survival (Figure 1g). These data suggest that JAK3-mutant T-ALL has a favorable response to the intensification of conventional cytotoxic chemotherapy in response to high levels of residual leukemia.

JAK3 mutations occurred at increased frequency in T-ALLs expressing the early T-cell precursor (ETP) immunophenotype (Figure 1h), consistent with previous reports (31), and ETP has been linked to poor clinical outcome in some studies (32, 33). However, ETP status was not significantly associated with mitochondrial apoptosis resistance (Supplementary Table 5). In a multivariable logistic regression model of treatment failure, ETP status was a significant predictor while JAK3 mutational status was not (P = 0.0001 for ETP and 0.71 for JAK3), indicating that JAK3 wild-type ETP T-ALLs harbor alternative mechanisms of treatment resistance. We recently reported that mutations of genes encoding polycomb repressive complex 2 (PRC2) subunits (EZH2, EED or SUZ12) induce apoptosis resistance in T-ALL (20), and both JAK3 and PRC2 mutations have been reported at high frequency in ETP T-ALL (31, 34, 35). However, JAK3 mutations were more strongly associated with apoptosis resistance than PRC2 mutations. Indeed, two-way ANOVA analysis including JAK3 and PRC2 mutational status revealed that only JAK3 mutations were associated with apoptosis resistance (P = 0.024). Thus, JAK3 mutations are associated with mitochondrial apoptosis resistance in childhood T-ALL.

Mutant JAK3 represses apoptosis in human leukemia cells.

To test whether JAK3 activating mutations directly regulate mitochondrial apoptosis, we focused on JAK3 mutant human T-ALL cells. None of the available T-ALL cell lines have an endogenous JAK3 mutation, thus we turned to two primary T-ALL cases expanded in immunodeficient mice as patient-derived xenograft (PDX) models, termed ETP1 and M1946E, which harbor JAK3 activating mutations that are either monoallelic (ETP1, an ETP T-ALL) or biallelic (M1946E, a non-ETP T-ALL) (Supplementary Figure 3). To

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inhibit JAK3, we leveraged two mechanistically distinct JAK3 small molecule inhibitors: i) tofacitinib, a JAK3 inhibitor FDA-approved for the treatment of autoimmune arthritis and colitis (36-39), and ii) ritlecitinib (also known as PF06651600), a covalent and highly selective inhibitor of JAK3 that is also in human clinical trials (40, 41). Importantly, while tofacitinib can inhibit JAK1 and JAK2 at higher doses, ritlecitinib has no such effect because it covalently binds to a cystine uniquely present in JAK3, and these two JAK3 inhibitors have non-overlapping off-target activity profiles (39, 40, 42).

Treatment with either tofacitinib or ritlecitinib significantly impaired viability of JAK3-mutant ETP1 and M1946E T-ALL PDX cells, but not the JAK3 wild-type control CBAT91163 (hereafter, CBAT) (Figure 2a). To test whether this was an apoptotic effect, we performed intracellular BH3 (iBH3) profiling analysis for cytochrome c release in PDX cells stimulated with a pro-apoptotic BIM BH3 peptide. The dose of BIM peptide was optimized for each PDX model to yield cytochrome c release rates in the linear part of the dose-response range. This revealed that tofacitinib or ritlecitinib treatment increased cytochrome c release in response to a fixed dose of pro-apoptotic BIM peptide in JAK3-mutant T-ALL cells (Figure 2b-c), indicating a reversal of mitochondrial apoptosis resistance. By contrast, tofacitinib had no effect on cytochrome c release in JAK3 wild-type CBAT cells (Figure 2b), arguing against nonspecific toxicity. We confirmed that tofacitinib or ritlecitinib treatment induced caspase activation and annexin V positivity in JAK3 mutant, but not in JAK3 wild-type, T-ALL cells (Figure 2d-e), confirming induction of apoptosis. Thus, JAK3 inhibition reverses apoptosis resistance in JAK3-mutant T-ALL.

JAK3 regulates BCL2 phosphorylation in ETP T-ALL.

We then asked how JAK3 regulates mitochondrial apoptosis, which is controlled by the relative activity of pro- and anti-apoptotic proteins of the BCL2 family (43). JAK3 is best-known for its ability to phosphorylate the STAT family of transcription factors, and JAK-STAT signaling has been shown to regulate mRNA expression of a number of pro- and anti-apoptotic BCL2 family members in some contexts, including BCL2, MCL1, BCL-XL, BIM, BID and BAD (44-48). While pharmacologic JAK3 inhibition blocked phosphorylation of multiple STAT proteins in JAK3-mutant T-ALL (Figure 3a), this resulted in only subtle changes in mRNA expression of BCL2 family genes (Supplementary Figure 4), and no measurable changes in protein levels of key BCL2 family members (Figure 3b). These findings argue that, in T-ALL, the effects of JAK3 signaling on mitochondrial apoptosis are not mediated by altered expression of BCL2 family members.

Aside from its ability to control STAT-mediated transcription, JAK3 also activates signal transduction cascades downstream of cytokine receptor signaling. In hematopoietic cells, cytokine signaling has been shown to repress mitochondrial apoptosis by stimulating BCL2 Ser70 phosphorylation to potentiate its antiapoptotic activity (49, 50). This phosphorylation has been shown to be mediated the kinases ERK1/2 (also known as MAPK3/1) (51), and MEK/ERK signaling is known to be activated by JAK3 in T-ALL (52). Indeed, we found that JAK3 inhibition with either tofacitinib or ritlecitinib inhibited MEK, ERK, and BCL2 Ser70 phosphorylation in JAK3-mutant T-ALL (Figure 3c). If JAK3 regulates T-ALL viability and mitochondrial apoptosis through this pathway, then a number of testable

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predictions emerge from this model, which we tested as follows. First, we found that treatment with the selective MEK inhibitor selumetinib (53) inhibited viability in PDX cells from the JAK3-mutant cases ETP1 and M1946E, but not in the JAK3 wild-type control CBAT (Figure 3d). Additionally, we found that selumetinib treatment phenocopied the ability of JAK3 inhibition to increase mitochondrial apoptotic priming, as assessed by flow cytometry iBH3 profiling analysis, in JAK3-mutant T-ALL (Figure 3e). Finally, if JAK3 inhibits mitochondrial apoptosis by phosphorylation and activation of BCL2, then primary JAK3-mutant T-ALL specimens should be characterized by BCL2 dependence. To test this prediction, we took advantage of the fact that the fluorimeter-based BH3 profiling assay we used to analyze the cohort of primary T-ALL specimens in our study can be used to classify these cases as BCL2 vs. BCL-XL dependent based on the pattern of response to BAD and HRK peptides (54, 55). These two peptides inhibit BCL-XL with similar affinities, and thus give a roughly equal signal in cells that are BCL-XL dependent. By contrast, the BAD peptide inhibits BCL2 more effectively than the HRK peptide, thus BCL2-dependent cells are characterized by a stronger response to BAD than HRK. Using our previously defined cutoffs (54), we found that 8 of the 10 JAK3-mutant primary T-ALL specimens were characterized by BCL2 dependence (Figure 3f; $P = 0.0481$). Taken together, these data support the model that JAK3 represses mitochondrial apoptosis in T-ALL via a MEK/ERK mediated signal transduction pathway that culminates in phosphorylation of BCL2 to stimulate its antiapoptotic activity.

Tofacitinib treatment synergizes with corticosteroids.

Our data, together with recently published results (27), support the therapeutic potential of JAK3 inhibitors in JAK3-mutant T-ALL. However, monotherapy with kinase inhibitors almost invariably leads to the development of resistance (56). To identify rational therapeutic combinations, we tested the therapeutic potential of the FDA-approved JAK3 inhibitor tofacitinib in combination with a panel of chemotherapeutics that are used in standard-of-care therapy for newly diagnosed or relapsed T-ALL, in JAK3-mutant PDX T-ALL cells. Bliss independence model analysis (57) revealed that tofacitinib was strongly synergistic (excess over bliss > 10) with the corticosteroids dexamethasone and prednisolone (Figure 4a-b and Supplementary Figure 5-6). Tofacitinib had largely additive efficacy when combined with the other tested chemotherapeutics (Figure 4a).

Treatment of ETP1 PDX T-ALL cells, which are JAK3-mutant, with tofacitinib in combination with dexamethasone revealed significantly more induction of apoptosis than treatment with either drug alone, as assessed by caspase activation or annexin V/PI staining (Figure 4c-d). This combination also showed significantly increased cytotoxicity to these cells than either drug alone (Figure 4e). We confirmed these effects in an independent JAK3-mutant T-ALL PDX, M1946E, revealing strong induction of apoptosis in response to the combination of tofacitinib and dexamethasone (Figure 4f-h).

To test the *in vivo* activity of this combination, we injected JAK3-mutant ETP1 PDX cells into sub-lethally irradiated NSG mice. At the time of leukemic engraftment, as assessed by detectable human leukemic blasts in the peripheral blood, mice were randomized into treatment groups (Figure 5a). Treatment was delivered continuously for 2 weeks by osmotic

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pump containing vehicle, tofacitinib (50 mg/kg/day), dexamethasone (0.2 mg/kg/day), or both drugs in combination, and mice were harvested and tumor burden was assessed on day 15. Treatment was well-tolerated, with the combination having no more effect on mouse weights than dexamethasone alone (Figure 5b). Consistent with our predictions, we found that the combination of tofacitinib and dexamethasone was significantly more effective than either therapy alone, as assessed by measurements of leukemic burden in the peripheral blood and the spleen (Figure 5c-f).

DISCUSSION

Here we show that JAK3 activating mutations are associated with resistance to mitochondrial apoptosis, as well as with a poor response to induction chemotherapy, in childhood T-ALL. Inhibition of JAK3 reverses the mitochondrial apoptotic blockade in JAK3 mutant leukemias. Our data support a model whereby JAK3 activating mutations inhibit apoptosis via a signal transduction pathway involving MEK and ERK that culminates in phosphorylation of BCL2 to stimulate its antiapoptotic activity. Consistent with this model, primary JAK3-mutant T-ALL patient samples were characterized by a dependence on BCL2. Because kinase inhibitor monotherapy in genetically complex tumors typically leads to the rapid development of resistance (56), we reasoned that the use of JAK3 inhibitors for T-ALL therapy with curative intent will require rational combinations. We thus tested the activity of JAK3 inhibition in combination with a panel of chemotherapeutics with known clinical activity in T-ALL. This revealed that JAK3 inhibition is potently synergistic with glucocorticoids, suggesting that the recently described link between IL7 receptor signaling and steroid resistance is mediated, at least in part, via JAK3 (58-60).

The ability of tumor cell mitochondria to resist pro-apoptotic stimuli has emerged as a cellular phenotype that robustly predicts chemotherapy resistance, thus revealing a prognostic biomarker that could further refine contemporary risk-adapted therapy. However, current strategies to intensify therapy in response to poor prognostic markers have been heavily biased towards the delivery of higher doses of conventional cytotoxic therapies and their associated toxicities. Fully exploiting the potential of BH3 profiling to improve clinical outcomes will require defining the pathobiology underlying this apoptotic phenotype, and translating these findings into rational therapeutic interventions. Our findings demonstrate that JAK3 mutations are directly responsible for resistance to mitochondrial apoptosis in T-ALL. These findings, together with the data that inhibition of JAK-STAT pathway alterations has therapeutic activity in T-ALL (27, 34, 52), provide strong support for clinical trials combining JAK3 inhibition with corticosteroids for the treatment of JAK3-mutant T-ALL. We also note that 5 of 6 JAK3 wild-type ETP T-ALLs were associated with BCL2 dependence by BH3 profiling in our cohort. Given that JAK3 wild-type ETP T-ALLs frequently harbor alternative mutations that activate cytokine and MAPK signaling, such as *IL7R, FLT3* and *JAK1* (31, 58, 60), and that this pathway can also be activated in T-ALL cells by physiologic IL7 ligand (59), it will be of interest to investigate whether overlapping pathobiology might underlie treatment failure in these cases.

The traditional approach to introduce new agents for the treatment of most cancers has been to do so in the context of relapsed/refractory disease. However, given that clinical outcomes

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for relapsed T-ALL are poor despite administration of therapy that is highly toxic (18, 19), we envision that the greatest impact of this combination will be as part of a biomarker-driven strategy to incorporate molecularly targeted therapies for patients at particularly high risk of relapse. Indeed, supplementing existing mutational and minimal residual disease-based biomarkers with functional biomarkers of treatment resistance, such as BH3 profiling, will allow patients at particularly high risk of treatment failure to be prioritized for innovative clinical trials, which are urgently needed for patients with high-risk T-ALL. Our studies provide a molecular rationale for clinical trials testing JAK3 inhibitors as a strategy to overcome early treatment resistance in the JAK3-mutant subset of ETP T-ALL.

Supplementary Material

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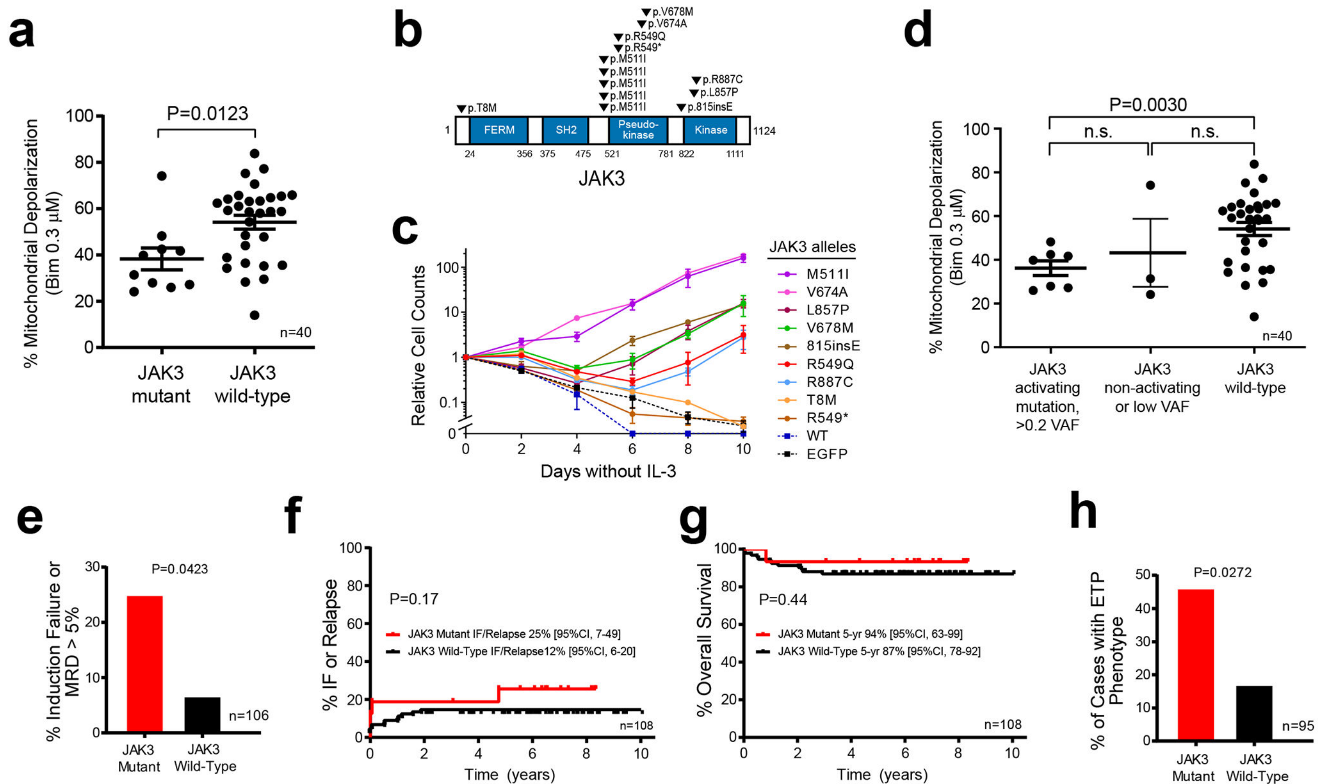

Fig. 1. JAK3 mutations are associated with resistance to mitochondrial apoptosis in human T-ALL.

a Association of JAK3 genotype with percent mitochondrial depolarization by BH3 profiling (fluorimeter-based assay) in primary pediatric T-ALL patient samples taken at initial diagnosis. Each data point represents mitochondrial depolarization in an independent T-ALL sample. P value calculated with a two-sided Welch t-test.

b Nonsynonymous protein-coding mutations of JAK3 identified in T-ALL cases analyzed by both BH3 profiling and sequencing analysis. Amino acid numbering based on NM_000215, and domain boundaries based on uniprot entry P52333.

c Ba/F3 cells were transduced with the indicated JAK3 alleles, or EGFP negative control, and then IL3 was withdrawn. Cell counts were assessed at the indicated time points by trypan blue vital dye exclusion staining and normalized to Day 0.

d Association of JAK3 genotype with percent mitochondrial depolarization by BH3 profiling (fluorimeter-based assay) in primary pediatric T-ALL patient samples taken at initial diagnosis. Each data point represents mitochondrial depolarization in an independent T-ALL sample. Data are shown by JAK3 mutation type, comparing cases with JAK3 mutations that were activating (based on ability to rescue Ba/F3 cells from cytokine withdrawal) and present at high variant allele fractions (VAF > 0.2), JAK3 mutations that were not activating or that were present at low variant allele fractions, or JAK3 wild-type cases. Significance was assessed by a two-sided Welch t-test with a Bonferroni correction for multiple comparisons.

e Association of JAK3 mutation status with response to induction chemotherapy. A poor response to induction was defined as protocol-defined induction failure (IF), or equal or more than 5% minimal residual disease (MRD), at the end of the first month of intensive

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induction chemotherapy. Note that this cohort includes T-ALL cases analyzed by BH3 profiling in Fig. 1a and 1b, well as an additional cohort of T-ALL samples sequencing analysis on which BH3 profiling data was not available (see Table S1). P value calculated by Fisher exact test. Number of samples per group: JAK3 mutant, n = 16; JAK3 wild-type n = 90. Note that for one patient outcome data were not available.

f Comparison of cumulative incidence of induction failure (IF) or relapse among T-ALL cases by JAK3 mutation type was calculated from the date of study enrollment to the event with induction/remission/toxic death as a competing risk and analyzed using the Gray test. Number of samples per group: mutants, n = 16; WT, n = 92. Note that for one patient outcome data were not available.

g Comparison of overall survival among JAK3 wild-type versus mutant T-ALL. Significance assessed by log-rank test. Number of samples per group: mutants, n = 16; WT, n = 92.

h Association of JAK3 mutation type with early T-cell progenitor (ETP) immunophenotype. P value calculated by Fisher exact test. Number of samples per group: JAK3 mutant, n = 13; JAK3 wild-type, n = 82. Note that ETP status was not available on a subset of cases (n = 14) analyzed in (d).

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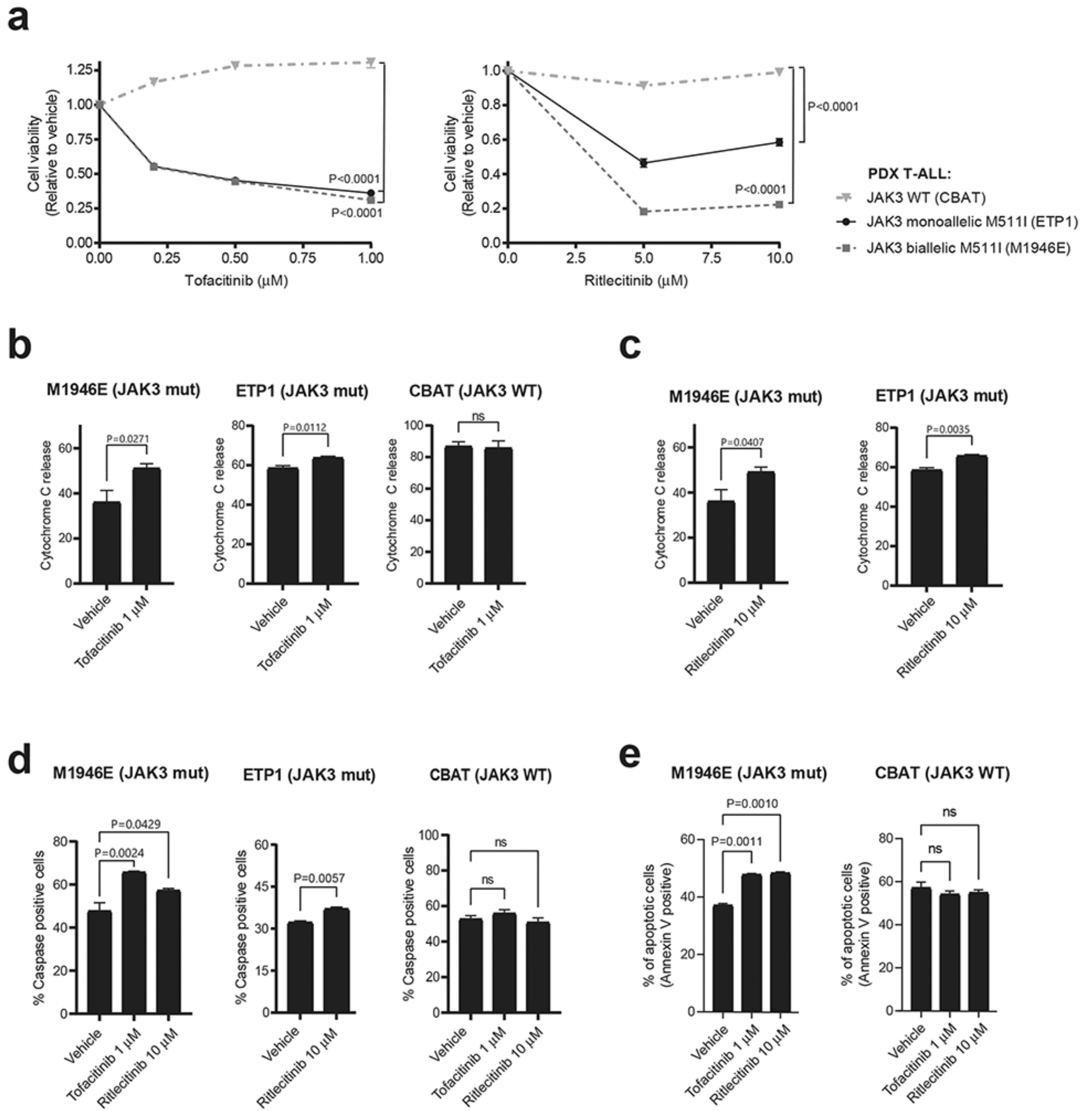

Fig. 2. JAK3 activating mutations repress mitochondrial apoptosis in T-ALL.

a JAK3 mutant PDX T-ALL cells (ETP1 or M1946E), or JAK3 wild-type PDX T-ALL cells (CBAT), were treated with the JAK inhibitors tofacitinib or ritlecitinib for 48 hours, and viability was assessed using CellTiter-Glo. Significance was assessed with a two-way ANOVA with a Dunnett correction for multiple comparisons.

b The indicated T-ALL PDX cells were treated with 1 μM tofacitinib for 6 hours. FACS-based iBH3 profiling was then performed to assess the degree of mitochondrial apoptosis resistance, based on cytochrome c release following treatment with pro-apoptotic BIM peptide for 40 minutes. Significance was assessed with a one-way Welch t-test.

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c The indicated PDX T-ALL cells were treated with 10 μM ritlecitinib for 6 hours. FACS-based iBH3 profiling was then performed to assess the degree of mitochondrial apoptosis resistance, based on cytochrome c release following treatment with pro-apoptotic BIM peptide for 40 minutes. Significance was assessed with a one-way Welch t-test.
d The indicated PDX T-ALL cells were treated with 1 μM tofacitinib or 10 μM ritlecitinib for 24 hours, and caspase activation was assessed by flow cytometry following staining with a fluorescent FAM-VAD-FMK probe that binds activated caspases (Image-iT LIVE caspase detection assay). The total amount of caspase+ cells in the human live (PI staining negative) population was graphed. Significance was assessed by one-way ANOVA with a Dunnett correction for multiple comparisons.
e The indicated PDX T-ALL cells were treated with 1 μM tofacitinib or 10 μM ritlecitinib for 24 hours, and apoptosis was assessed using annexin V/propidium iodide (PI) staining. The total % of Annexin V positive human cells was graphed. Significance was assessed by one-way ANOVA with a Dunnett correction for multiple comparisons.
Results shown represent the mean ± SEM of at least n = 3 biological replicates from one representative experiment, all of which were repeated independently at least twice.

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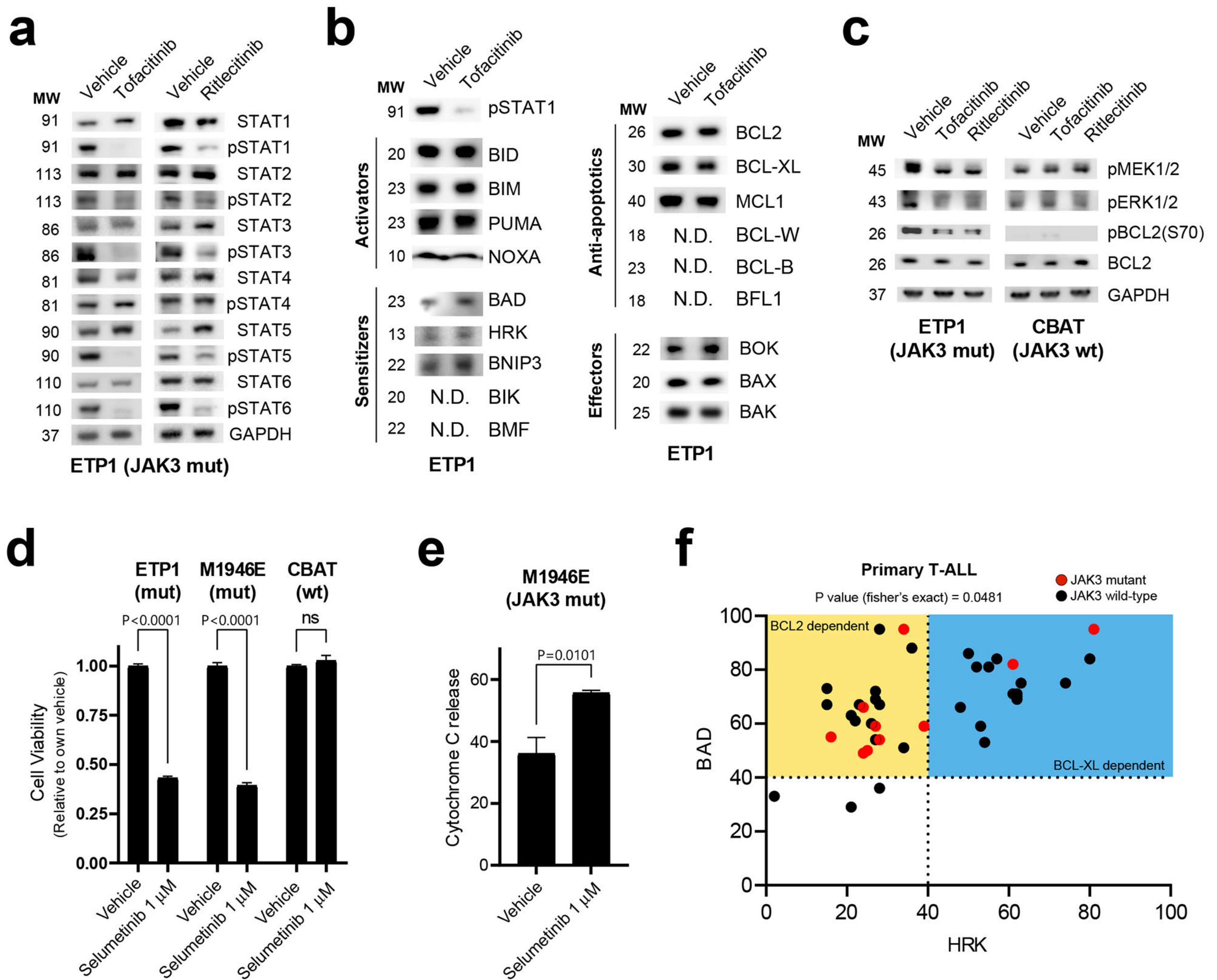

Fig 3. JAK3 positively regulates phosphorylation of MEK, ERK and BCL2, and JAK3-mutant primary T-ALLs are characterized by BCL2 dependence.

a-c The indicated PDX T-ALL cells were treated with 1 μM tofacitinib or 10 μM ritlecitinib for 24 hours, and western blotting with the indicated antibodies was performed with 30 μg protein lysate loaded in every lane. Note that there was no detection of pBCL2 in the JAK3 wild-type WT PDX CBAT when exposed with the strongest substrate and longest possible exposure time. MW, molecular weight. N.D., not detected.

d JAK3 mutant PDX T-ALL cells (ETP1 or M1946E), or JAK3 wild-type PDX T-ALL cells (CBAT), were treated with 1 μM selumetinib (a MEK inhibitor) for 48 hours, and viability was assessed using CellTiter-Glo. Significance was assessed by a two-sided Welch t-test with a Bonferroni correction for multiple comparisons.

e The indicated PDX T-ALL cells were treated with 1 μM selumetinib for 6 hours. FACS-based iBH3 profiling was then performed to assess the degree of mitochondrial apoptosis resistance, based on cytochrome c release following treatment with pro-apoptotic BIM peptide for 40 minutes. Significance was assessed with a one-way Welch t-test.

f BCL2 versus BCL-XL dependency of primary pediatric T-ALL patient samples was assessed by comparing their relative sensitivity to BAD vs HRK peptide, as assessed

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using fluorimeter-based BH3 profiling analysis. Each data point represents mitochondrial depolarization in an independent T-ALL sample. Colors of each dot indicate the JAK3 genotype. Significance was assessed using a one-sided fisher's exact test.

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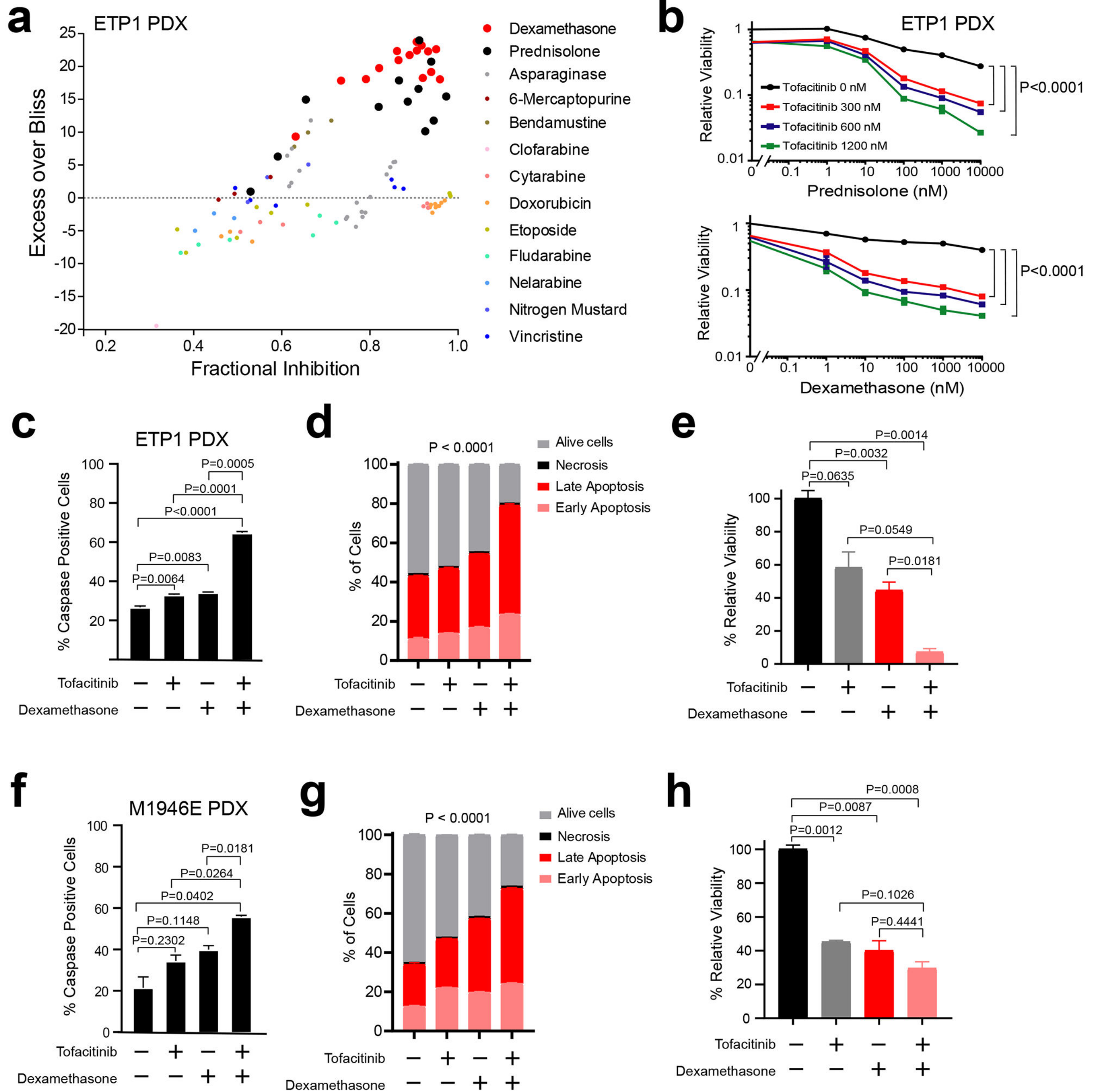

Fig 4. Tofacitinib treatment synergizes with corticosteroids.

a Synergy testing was performed using Bliss independence model analysis of viability data of ETP1 PDX T-ALL cells treated with tofacitinib in combination with a range of doses of the indicated chemotherapeutics for 48 hours, and viability was assessed using CellTiter-Glo. Data are shown for dose combinations resulting in fractional inhibition of 0.2 – 0.98. Note strongly positive Excess over Bliss (>10) of tofacitinib with prednisolone and dexamethasone, indicating synergistic interactions. Each dot represents the mean of experimental triplicates of a single dose combination.

b Viability curves of ETP1 PDX cells treated with tofacitinib in combination with prednisolone or dexamethasone used for Bliss independent model analysis in (a).

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Logarithmic graphing of the y-axis is chosen to capture the synergistic effects of the combination treatment; note that the viability effects of tofacitinib alone are comparable with data in Figure 2. Significance was assessed with a two-way ANOVA with a Dunnett correction for multiple comparisons.

c ETP1 PDX cells were treated with vehicle, 1 μM tofacitinib, 50 nM dexamethasone, or both drugs in combination for 24 hours, and caspase activity was assessed by flow cytometry following staining with a fluorescent FAM-VAD-FMK probe that binds activated caspases (Image-iT LIVE caspase detection assay). The total amount of caspase positive cells in the human live (PI staining negative) population was graphed. A one-way ANOVA with a Dunnett correction for multiple comparisons was used for statistical analysis.

d ETP1 PDX cells were treated for 24 hours with the indicated drugs as in (c), and apoptosis was assessed using annexin V/propidium iodide (PI) staining. Significance was assessed by two-way ANOVA with a Dunnett correction for multiple comparisons). Note $P < 0.0001$ for all treatment conditions based on the total (late + early) apoptotic cells.

e ETP1 PDX cells were treated with the indicated drugs as in (c), and viability was assessed after 4 days of treatment based on trypan blue cell exclusion. Significance was assessed with a one-way ANOVA with a Dunnett correction for multiple comparisons.

f-h Cells from an additional independent JAK3-mutant PDX (M1946E) were treated with the indicated drugs, and the indicated markers of apoptosis or viability were assessed as described in (c-e).

Results shown in (b-h) represent the mean ± SEM of at least n = 3 biological replicates from one representative experiment, and each experiment was repeated independently at least twice.

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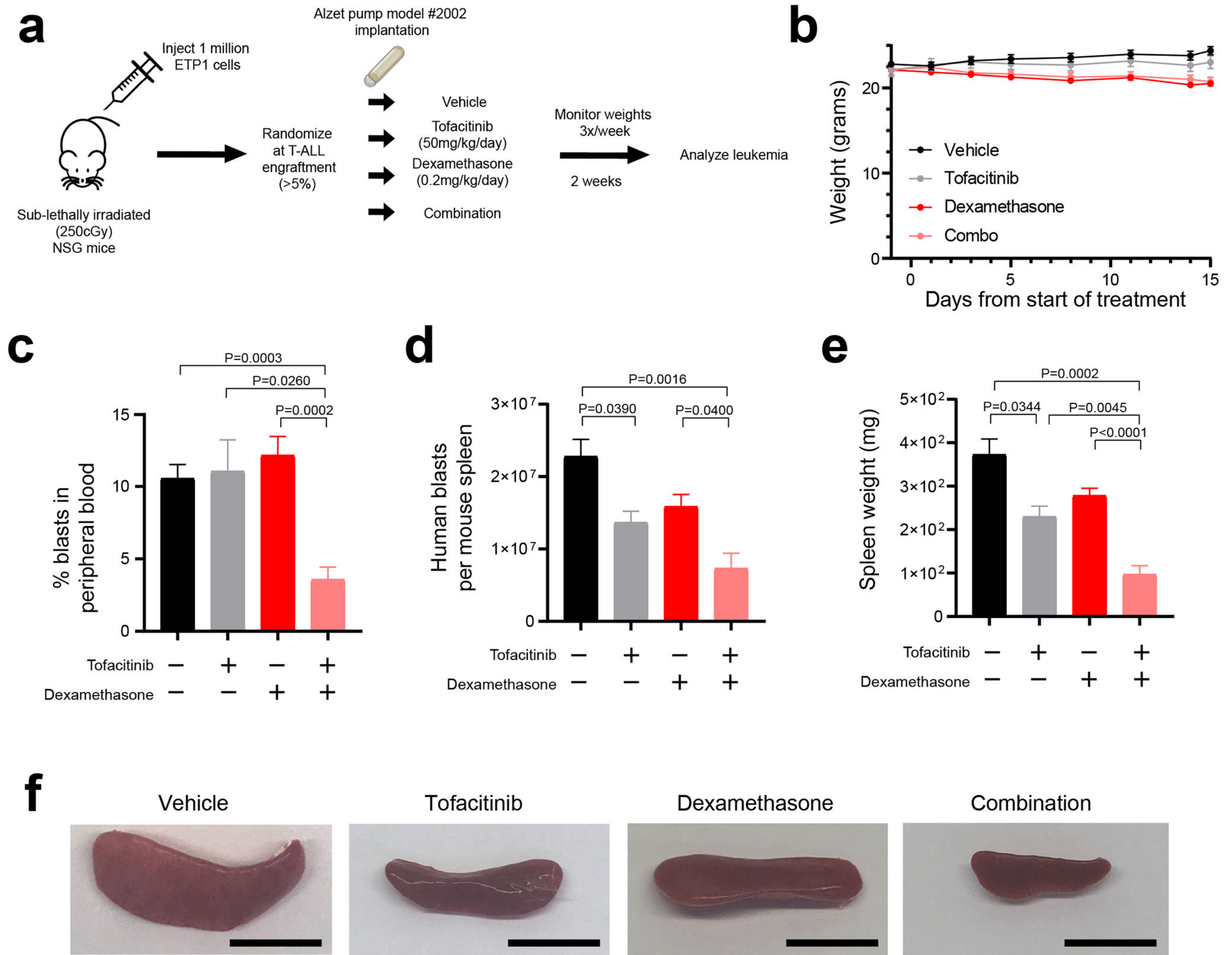

Fig 5. In vivo activity of the combination of tofacitinib and dexamethasone in human JAK3-mutant T-ALL.

a Experimental design. Human ETP1 PDX cells were injected into NSG immunodeficient mice after sublethal irradiation. Once engraftment of leukemia (>5% human leukemic cells in peripheral blood) was confirmed, mice were implanted with subcutaneous osmotic pumps that delivered the indicated daily dose of each drug over 2 weeks. Leukemic burden was analyzed 2 weeks after treatment start.

b Weights of mice throughout treatment course.

c Peripheral blood was obtained from mice on day 13 of treatment, and the percentage of human leukemic blasts was assessed by flow cytometry following red blood cell lysis and staining with human CD45 antibody. Significance was assessed with a one-way ANOVA with a Dunnett correction for multiple comparisons.

d Percentage of human blasts per mouse spleen was assessed by flow cytometry following staining with human CD45 antibody. Significance was assessed with a one-way ANOVA with a Dunnett correction for multiple comparisons.

e Spleen weight at the end of treatment. A one-way ANOVA with a Dunnett correction for multiple comparisons was used for statistical analysis.

f Representative images of spleens harvested from mice two weeks post treatment. Scale bar indicates 10mm.

Results shown in (b-e) represent the mean ± SEM of at least n = 7 mice per group.

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