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Enhanced MAPK signaling induced by CSF3Rmutants confers dependence to DUSP1 for leukemic transformation

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Abstract:
Elevated MAPK and the JAK-STAT signaling play pivotal roles in the pathogenesis of chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML). While inhibitors targeting these pathways effectively suppress the diseases, they fall short in providing enduring remission, largely attributed to cytostatic nature of these drugs. Even combinations of these drugs are ineffective in achieving sustained remission. Enhanced MAPK signaling besides promoting proliferation and survival triggers a pro-apoptotic response. Consequently, malignancies reliant on elevated MAPK signaling employ MAPK-feedback regulators to intricately modulate the signaling output, prioritizing proliferation and survival while dampening the apoptotic stimuli. Herein, we demonstrate that enhanced MAPK signaling in CSF3R (Granulocyte-colony stimulating factor receptor)-driven leukemia upregulates the expression of Dual specificity phosphatase 1 (DUSP1) to suppress the apoptotic stimuli crucial for leukemogenesis. Consequently, genetic deletion of Dusp1 in mice conferred synthetic lethality to CSF3R-induced leukemia. Mechanistically, DUSP1 depletion in leukemic context causes activation of JNK1/2 that results in induced expression of BIM and P53 while suppressing the expression BCL2 that selectively triggers apoptotic response in leukemic cells. Pharmacological inhibition of DUSP1 by BCI (a DUSP1 inhibitor) alone lacked anti-leukemic activity due to ERK1/2 rebound caused by off-target inhibition of DUSP6. Consequently, a combination of BCI with a MEK inhibitor successfully cured CSF3R-induced leukemia in a preclinical mouse model. Our findings underscore the pivotal role of DUSP1 in leukemic transformation driven by enhanced MAPK signaling and advocate for the development of a selective DUSP1 inhibitor for curative treatment outcomes.

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Enhanced MAPK signaling induced by CSF3R mutants confers dependence to DUSP1 for leukemic transformation

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1. Genetic deletion of *Dusp1* eliminates CSF3R-induced leukemia.
2. Inhibition of Dusp1 induces the expression of Bim and p53 in oncogenic-context resulting to selective demise of leukemic cells.

Abstract

Elevated MAPK and the JAK-STAT signaling play pivotal roles in the pathogenesis of chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML). While inhibitors targeting these pathways effectively suppress the diseases, they fall short in providing enduring remission, largely attributed to cytostatic nature of these drugs. Even combinations of these drugs are ineffective in achieving sustained remission. Enhanced MAPK signaling besides promoting proliferation and survival triggers a pro-apoptotic response. Consequently, malignancies reliant on elevated MAPK signaling employ MAPK-feedback regulators to intricately modulate the signaling output, prioritizing proliferation and survival while dampening the apoptotic stimuli. Herein, we demonstrate that enhanced MAPK signaling in CSF3R (Granulocyte-colony stimulating factor receptor)-driven leukemia upregulates the expression of Dual specificity phosphatase 1 (DUSP1) to suppress the apoptotic stimuli crucial for leukemogenesis. Consequently, genetic deletion of *Dusp1* in mice conferred synthetic lethality to CSF3R-induced leukemia. Mechanistically, DUSP1 depletion in leukemic context causes activation of JNK1/2 that results in induced expression of BIM and P53 while suppressing the expression BCL2 that selectively triggers apoptotic response in leukemic cells. Pharmacological inhibition of DUSP1 by BCI (a DUSP1 inhibitor) alone lacked anti-leukemic activity due to ERK1/2 rebound caused by off-target inhibition of DUSP6. Consequently, a combination of BCI with a MEK inhibitor successfully cured CSF3R-induced leukemia in a preclinical mouse model. Our findings underscore the pivotal role of DUSP1 in leukemic transformation driven by enhanced MAPK signaling and advocate for the development of a selective DUSP1 inhibitor for curative treatment outcomes.

Introduction

Enhanced mitogen activated protein kinases (MAPK) activity due to activating mutations or overexpression of pathway components is one of the hallmarks of cancers[1]. Despite considerable knowledge about MAPK interactions with downstream effectors, the intricacies driving cellular transformation remain less elucidated. Depending on the cellular and genetic context, MAPK signaling can either promote cell proliferation and survival or instigate apoptotic machinery for cellular destruction[2,3]. For instance, RAS-MAPK signaling not only triggers proliferative pathways but also activates stress-activated MAPKs, P38, and c-Jun N-terminal kinase (JNK), implicated in apoptosis induction[4,5]. However, the fate of cells; whether they undergo apoptosis or proliferate, is determined by genetic and cellular contexts. To accomplish this, most tumor cells employ MAPK negative-feedback regulators to suppress the apoptotic response[6]. Importantly, the magnitude, duration, and location of MAPK signaling are strictly regulated to support malignant growth[7]. Both positive and negative feedback regulators are implicated in shaping the final signaling-output through spatio-temporal regulation. MAPK negative feedback loops are comprised of both transcriptional (MAPK phosphatases or dual specificity phosphatases, DUSPs) and post-transcriptional (direct phosphorylation of pathway components by ERK1/2)[6]. While the mechanisms activating RAS-MAPK signaling in cancer cells are well-explored, the understanding of how negative feedback regulators contribute to cellular transformation and treatment outcomes is not fully understood. Given the essential role of MAPK negative regulators in dampening the apoptotic stimulants, we hypothesize that inhibiting these regulators could unleash robust apoptotic response that may selectively eliminates the leukemic clones.

Activating mutations in CSF3R (Granulocyte-colony stimulating factor receptor, G-CSF3R) have been reported in patients with chronic neutrophilic leukemia (CNL) and acute myeloid leukemia (AML)[8]. A minority of patients harbor more than one mutation (compound mutation), which causes more aggressive leukemia. Previous studies from Drucker and Tyner's group reported that CSF3R proximal mutants depend on JAK-STAT signaling, while the truncation mutants seemingly rely on SRC-dependent survival[8]. Despite significant strides in understanding the biology of CSF3R-induced leukemogenesis, effective treatment is lacking especially for high-risk CNL patients. Our earlier work using mouse models underscored the essential role of enhanced MAPK signaling in CSF3R-induced leukemia[9]. As a result, treatment with a MEK inhibitor suppressed the leukemic progression. However, akin to JAK2 inhibitors, MEK inhibition engendered a cytostatic response. Even a combination of inhibitors targeting both JAK2 and MEK was ineffective in inducing the clonal selectivity[9]. Subsequent studies, using unbiased phospho-proteomic analyses, unveiled persistent BTK signaling in CSF3R-induced leukemia[10]. Nevertheless, attempts to enforce clonal selectivity through inhibition of BTK alone or in combination with JAK2 or MEK inhibitors were ineffective (unpublished).

Cancers fueled by elevated MAPK signaling induce its negative feed-back regulators (MKP activity) to fine-tune the signaling output and suppress ERK-induced apoptotic activity to foster cancer progression[5,11,12]. Herein, we show that enhanced MAPK activity in CSF3R-induced leukemia is associated with elevated expression of Dual specificity phosphatase 1 (*Dusp1*). Deletion of *Dusp1* is synthetic lethal to CSF3R-induced leukemia. DUSP1 depletion in the leukemic context induced the enzymatic activity of JNK1/2 that suppressed the expression of BCL2 while inducing the expression of BIM and P53. This meticulous fine-tuning of apoptotic machinery resulted in selective demise of leukemic cells. Unexpectedly, chemical inhibition of DUSP1 by BCI was ineffective in suppressing the leukemic progression. We noted that ERK1/2 rebound abrogated the treatment response to BCI due to -off target inhibition of DUSP6. As a proof-of-concept, ectopic over-expression of *Dusp6* in leukemic cells restored anti leukemic

effect of BCI. Accordingly, leukemic mice treated with a combination of BCI and trametinib, like genetic deletion of *Dusp1*, selectively eradicated the CSF3R-induced leukemia. Altogether, these observations provide evidence that DUSP1 confers oncogene-dependence, while DUSP6 functions as a tumor suppressor in CSF3R-driven leukemia. Targeting DUSP1 with a selective inhibitor could provide a curative response to CSF3R-mutant driven myeloid leukemia.

Materials and Methods

Plasmids and inhibitors

Retroviral plasmids expressing CSF3R mutants (CSF3R-WT, CSF3R^{T618I}, CSF3R^{Q741*}, CSF3R^{W791*}, CSF3R$^{T618I/Q741*,}$ and CSF3R$^{T618I/W791}$) were described earlier[9]. Ruxolitinib (JAK2 inhibitor), and Trametinib (MEK1/2 inhibitor) were purchased from AdooQ Biosciences, USA. DUSP1 inhibitor BCI was custom synthesized by Tocris (Bio-Techne incorporation, USA).

Cell lines

BaF3 cell line, a growth factor dependent murine pro-B cell line, was transduced using retroviral vectors expressing CSF3R mutants and venus as described earlier[13,14]. After 24 hrs of viral transduction, venus positive cells were sorted by FACS(Fluorescence-Activated Cell Sorting) and grown without IL3. Retroviral production and transduction were performed as described earlier[9]. BaF3 cells were grown in RPMI supplemented with 10% FBS and 100 µg/ml streptomycin +100 IU/ml penicillin, + 2mM L-glutamine. HEK293T were grown in DMEM containing 10% FBS and 100 IU/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine. For in vitro proliferation assays, BaF3 parental cells or BaF3 cells expressing CSF3R mutants were grown in RPMI supplemented with 10% FBS, 100 µg/ml streptomycin +100 IU/ml penicillin, 2mM L-glutamine, recombinant murine IL3 (10 ng /ml) from Peprotech (USA) for one week. All cell lines were evaluated for mycoplasma contamination.

Immunoblotting

Six million BaF3 cells expressing CSF3R variants were suspended in lysis buffer followed by sonication. Composition of lysis buffer has been described earlier[9]. Lysates were separated by 10% SDS-PAGE and transferred to supported nitrocellulose membrane (Bio-rad, USA) and probed with Anti-HA tag, Phospho-MEK1/2, phospho-ERK1/2, Phospho-STAT3, Phospho-StTAT5 MEK1/2, ERK1/2, STAT5, P53, BIM, BCL2, phospho-JNK1/2, JNK1/2, phospho-P38 , and P38 antibodies (Cell Signaling Technology, USA). Anti-DUSP1 antibody was purchased from Santacruz biotechnology, USA. All primary antibodies were used at dilutions as recommended by the manufacturer. Anti-mouse or anti-rabbit IgG HRP conjugated secondary antibodies (GE Healthcare, USA) were used at a 1:5000 dilution. HRP conjugated β-Actin antibody was purchased from the Cell Signaling Technology, USA. Immunoblots were developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, USA) followed with scanning on ChemiDoc^{TM} touch Imaging system (Bio-Rad, USA). All western blots were replicated twice.

Hematopoietic Colony forming cell assays.

Hematopoietic progenitors Kit^{+} cells from the bone marrow of C57Bl/6 or *Dusp1*$^{-/-}$ mice were isolated using the CD117 MicroBead Kit (Miltenyi biotec, inc, USA) according to the manufacturer's instructions. Cells were cultured in IMDM supplemented with 10% FBS and 100 µg/ml streptomycin +100 IU/ml penicillin, + 2mM L-glutamine with the addiftion of mSCF (50 ng/mL), mTPO (50 ng/mL), mFLT3-L (20 ng/mL), mIL6 (10 ng/mL), mIL3 (10ng/mL) (R&D systems, USA). After 12 hours of stimulation cells were transduced with retroviruses expressing CSF3R mutants and venus using home-made retronectin. Three-five thousand venus positive

cells (isolated by FACS) were plated in triplicate in MethoCult™ GF M3434 (STEMCELL Technologies, USA) with Ruxolitinib [1 μM], Trametinib [5 nM], BCI [400 nM], Ruxolitinib+BCI [1μM+400 nM], Trametinib+BCI [5nM+400 nM]. Colonies were enumerated after 1 week of incubation at 37°C.

Bone marrow transduction and transplantation

Bone marrow transduction and transplantations were performed as described earlier[9]. Kit+ bone marrow cells were isolated from the wild-type (C57Bl/6) or *Dusp1*$^{-/-}$ mice and transduced with retroviruses expressing vector (*pMSCV-Ires-Venus*, *pMIV*), *pMSCV-CSF3R*^{T618I}*-Ires-Venus*, and *pMSCV-CSF3R*$^{T618I/Q741*}$*-Ires-Venus*. Five mice for each construct were transplanted with one hundred thousand venus positive cells mixed with 0.2-0.5 million-helper bone marrow cells into each lethally irradiated mouse through tail vein injection. Sample sizes were chosen on the basis of previous experience and published transplantation data. The experiment was repeated three times with similar results. After two weeks of transplantation engraftments were determined by analyzing the venus positive cells from the peripheral blood using FACS. These mice were monitored for leukemia progression and survival, leukemic burden (venus positive cells) and white blood cell (WBC) numbers were determined weekly up to 16-18 weeks in surviving mice. All mouse work was performed with approval of the Institutional Animal Care and Use Committee (IACUC).

Flow Cytometry

Twenty microliters of peripheral blood (PB) were collected from the transplanted mice via tail bleeding. Cells were lysed using RBC lysis buffer (BD Technologies, USA) and the total mononuclear cells (TMNCs) were pelleted by centrifugation. The cell pellets were washed once with cold PBS followed with blocking for 10 minutes at room temperature using mouse FcR blocking reagent (Miltenyi biotec, inc, USA). Antibody staining and FACS analysis were performed as described earlier[9].

Drug preparation and in vivo treatments

All drug stocks were made in DMSO to 10 mM and stored in -20C until use. For *in vivo* injection, DMSO drug stocks of Ruxolitinib, Trametinib and BCI were diluted in PBS. After two weeks of transplantation, venus percentage was measured to determine the leukemic engraftment and chimerism. The mice were grouped, and drugs were orally administered or injected through i.p injection. Ruxolitinib (50 mg/kg twice a day) and Trametinib (10 mg/kg once daily) was given by oral gavage. BCI (10 mg/kg twice) was administered through i.p. injection as described earlier[14].

Statistical Analysis

Statistical analyses were performed using Prism software v9.0 (GraphPad Software, USA). The median survival was calculated by log-rank test. For *in vitro* studies, statistical significance was determined by the two-tailed unpaired Student's *t*-test. A p value < 0.05 was considered statistically significant. For all figures, NS, not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Unless otherwise indicated, all data represent the mean ± standard deviation (SD) from three technical replicates.

Results

Induced expression of *Dusp1* in CSF3R-induced leukemia

Earlier, we have shown that the expression of CSF3R wild-type or truncation mutants (Q741* or W791*) were unable to induce leukemia despite persistent granulocytic hyperplasia[9]. A comparative whole genome expression profiling of leukemic and non-leukemic CSF3R mutants revealed induced expression of 23 MAPK pathway genes in leukemic cells[9]. Consequently, MAPK negative regulators are induced to prevent the apoptotic stimulation associated with elevated MAPK activity. While we noted differential expression of Dusp family members in cells expressing CSF3R mutants compared to vector control, expression of *Dusp1* is specifically induced only in cells expressing CSF3R mutants (**Figure 1A**). Quantitative expression analysis by q-PCR of Lineage-, Kit+ and Sca1+ (LSK) cells revealed significant induction of *Dusp1* in cells expressing CSF3R variants compared to CSF3R-WT and vector control (**Figure 1B**). Notably, cells expressing leukemic CSF3R variants displayed higher *Dusp1* expression (**Figure 1B**). Next, to determine the role of *Dusp1* in leukemic transformation by CSF3R mutants, BaF3 cells expressing control *Sc-shRNA* and *Dusp1-shRNA*[14] were transduced with retroviruses expressing CSF3R mutants followed with analysis of growth kinetics and downstream MAPK signaling. The expression of CSF3R mutants renders BaF3 cells to growth factor independence although with differing transformation potential, as follows: compound mutations>proximal mutation>truncation mutation>wild-type[9]. As reported earlier[14], expression of *Dusp1-shRNA* resulted in ~60-70 % knockdown of DUSP1 protein (**Figure 1C**). Depletion of *Dusp1* did not show any effect on survival and proliferation of parental BaF3 or BaF3 cells expressing CSF3R mutants when grown with IL3 (**Figure 1D and Supplementary Figure 1A)**. However, in the absence of growth factor, IL3, *Dusp1* depletion significantly reduced the survival and proliferation of CSF3R mutants **(Figure 1E and Supplementary Figure 1B**). Biochemical analysis of MAPK signaling by western blotting revealed that DUSP1 depletion resulted in enhanced JNK1/2 activation and suppression of pERK1/2 compared to controls (cells expressing vector and non-leukemic CSF3R truncation mutants, $CSF3R^{Q741*}$ and $CSF3R^{W791*}$). In contrast, induction of phospho-P38 levels were noted indiscriminately in all CSF3R mutants, leukemic and non-leukemic (**Figure 1C**). Altogether, these results suggest that DUSP1 confers dependence to CSF3R-driven leukemia by modulating the MAPK-JNK1/2 signaling out-put.

Genetic deletion of *Dusp1* is synthetic lethal to CSF3R-induced leukemia.

While BaF3 cellular transformation and shRNA-knockdown studies are useful surrogate model for functional studies, it does not fully recapitulate *in vivo* disease development. To determine the role *Dusp1* in CSF3R-induced leukemogenesis*,* bone marrow derived Kit^+ cells from C57Bl/6 WT and *Dusp1*$^{-/-}$ mice were transduced with *CSF3R-Ires-venus* retroviruses for *in vitro* CFU assays and *in vivo* leukemogenesis (**Figure 2A**). Genetic deletion of *Dusp1* significantly reduced the CSF3R-induced CFUs (~ 90%) compared to vector control, cells expressing *MSCV-Ires-venus* (**Figure 2B**). As described earlier, CSF3R proximal ($CSF3R^{T618I}$) and compound mutants ($CSF3R^{T618I/Q741*}$) were used for *in vivo* leukemogenesis assay. Mice were transplanted with 80,000 venus positive *Dusp1* deficient and proficient Kit^+ cells expressing leukemic CSF3R mutants, $CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*}$, or *MSCV-Ires-venus* vector control. Mice transplanted with wild-type Kit^+ cells expressing leukemic CSF3R mutants developed fatal leukemia with a disease latency of 2–3 weeks for $CSF3R^{T618I/Q741*}$ and 13-15 weeks for the proximal-mutant $CSF3R^{T618I}$ (**Figure 2C-E**). Notably, mice recipients of *Dusp1* deficient Kit cells did not develop leukemia and exhibited disease-free survival (**Figure 2F-H)**. Strikingly, *Dusp1* deficient Kit cells expressing CSF3R mutants were gradually removed from the transplanted mice while control cells (expressing MSCV-Ires-venus vector) were maintained, suggesting that *Dusp1* deletion is synthetic lethal to CSF3R mutants (**Figure 2E and H**). Next, we performed secondary transplantation to confirm that the leukemic cells are completely eradicated from the bone marrow (BM) and mice were cured of the disease. Mice recipients of primary wild-type BM cells expressing CSF3R variants developed robust leukemia with a shorter disease latency (2-6 weeks) compared to primary transplantation. In contrast,

mice transplanted with *Dusp1* deficient primary BM cells exhibited leukemia-free survival without any trace of leukemic cells determined by venus positive cells (**Supplementary Figure 2A-H**). Altogether, these data provide evidence that *Dusp1* deletion confers synthetic lethality to CSF3R-induced leukemia.

Off-target Inhibition of DUSP6 abrogates the antileukemic response to DUSP1 inhibition by BCI.

To determine the potential of DUSP1 targeting, a small molecule DUSP1 inhibitor (BCI) was evaluated *in vitro* and *in vivo* assays either as a single agent or in combination with Jak2 inhibitor (Ruxolitinib). Primary bone marrow Kit^+ cells expressing CSF3R variants treated with BCI and Ruxolitinib alone indiscriminately suppressed the CFU formation in normal and leukemic cells (**Supplementary Figure 3**). However, a combination of BCI+Ruxolitinib showed significant suppression of CSF3R-induced CFUs compared to vector control (**Supplementary Figure 3**). Next, we examined the *in vivo* efficacy of BCI alone and with Ruxolitinib using the retroviral transduction and transplantation model described above. Drug treatments were started after two weeks of transplantation. As reported earlier, Ruxolitinib treatment suppressed the WBC levels but lacked clonal selectivity[9]. All transplanted mice showed progressive leukemia and eventually succumbed to the disease in both models of CSF3R-induced leukemia, $CSF3R^{T618I}$ (**Figure 3 A-C**) and $CSF3R^{T618I/Q741*}$ (**Figure 3 D-F**). Mice treated with BCI alone or in combination with Ruxolitinib did not show any improvement in leukemic progression or reduction in leukemic burden compared to the Ruxolitinib treatment group (**Figure 3A-F**). Altogether, these data suggest that treatment with BCI alone or in combination with Ruxolitinib is ineffective *in vivo*.

Next, we sought to understand how *Dusp1* deletion confers lethality to CSF3R mutants and why its chemical inhibition is ineffective. Because BCI inhibits both DUSP1 and DUSP6, we reasoned that inhibition of DUSP6 likely abolished DUSP1 dependence. We reasoned that a comprehensive analysis of DUSP1/DUSP6 substrates and their downstream targets implicated in mediating apoptosis (BIM, BCL2, and P53) will illuminate the underlying mechanisms driving synthetic lethality and why BCI treatment is ineffective (**Figure 4A** and **C**). Genetic knock-down of *Dusp1* revealed elevated p-JNK levels with a modest increase in p-P38 levels, while pERK1/2 levels were suppressed in cells expressing leukemic CSF3R variants compared to controls, pMIV-Sc-ShRNA and pMIV-Dusp1-ShRNA (**Figure 1C**). Consequently, proapoptotic protein BIM and P53 levels were significantly increased with a notable reduction in the levels of anti-apoptotic protein, BCL2, compared to Sc-shRNA control cells (**Figure 4B**). Because BCI also inhibits DUSP6, we reasoned that concomitant inhibition of DUSP6 would activate its preferred substrate ERK1/2 (**Figure 4C**). Elevated ERK1/2 activity beside promoting proliferation and survival modulates the turnover of BIM and BCL2[15-17]. ERK1/2 mediated phosphorylation of BIM is targeted for proteasome dependent degradation, while phosphorylation of BCL2 prevents its degradation[18,19]. As envisioned, BCI treatment resulted in increased phosphorylation of pERK1/2 with a marked reduction in p-JNK1/2, whereas p-P38 levels were unchanged (**Figure 4C and D**). In contrast to genetic inhibition of DUSP1, BCI treatment reduced BIM levels, suggesting that its turnover is directly controlled by ERK1/2. Similar to genetic inhibition of DUSP1, BCI treatment induced the expression of P53 while decreasing the BCL2 levels. Interestingly, cells treated with BCI+Trametinib exhibited greater P53 expression and suppression of BCL2 possibly due to restoration of JNK1/2 activity. Nonetheless, a negative crosstalk between ERK and JNK has been reported where sustained ERK activity suppressed JNK activation[20]. Next, leukemic cells were transduced with retroviruses expressing *Dusp6* to determine whether activation of ERK1/2 by BCI is due to inhibition of DUSP6 or some other MAPK phosphatases. Ectopic expression of *Dusp6* in primary BM cells significantly suppressed the CSF3R-induced CFUs compared to control (**Supplementary Figure 4 A**). Interestingly, BCI treatment of *Dusp6* overexpressing leukemic cells fully suppressed the CSF3R-dependent

CFUs (**Supplementary Figure 4A**). This suggests that inhibition of pERK1/2 may restore BCI sensitivity and result in selective eradication of leukemic cells, as noted with *Dusp1* deletion. Altogether these data provide evidence that off-target inhibition of DUSP6 by BCI resulted in higher ERK1/2 expression, which abrogated its antileukemic response. These results, suggest that inhibition of ERK1/2 may provide an effective response to BCI treatment.

A combination of BCI and Trametinib cured the mice of leukemia.

To test whether inhibition of ERK1/2 would restore the antileukemic efficacy of BCI, we performed *in vitro* CFU assays with Trametinib alone and in combination with BCI. As reported earlier, Trametinib alone suppressed the CSF3R mutant-induced CFUs. However, it also equally suppressed the control cells, expressing vector pMSCV-Ires-venus (**Supplementary Figure 4B**). Strikingly, the combination of BCI+Trametinib fully suppressed the CSF3R-induced CFUs without any noted toxicity for control cells compared to cells treated with Trametinib alone (**Supplementary Figure 4B**). Next, we examined the *in vivo* efficacy of BCI with Trametinib using retroviral transduction and transplantation model. After two weeks of transplantation, leukemic engraftments, and percent chimerism were determined by analyzing the venus-positive cells from the peripheral blood using FACS. Mice were randomized; five mice per group were treated with vehicle, Trametinib alone, and BCI+Trametinib for eight weeks. As reported earlier, treatment with Trametinib alone suppressed the leukemic burden and prolonged the survival of mice but lacked clonal selectivity. While Trametinib treatment prevented disease-related death in $CSF3R^{T618I}$ transplanted mice, its efficacy was significantly reduced against the compound mutant ($CSF3R^{T618I/Q741*)}$ as almost all treated mice succumbed to the disease. Strikingly, treatment with BCI+Trametinib for six weeks resulted in complete suppression of disease progression and rescued ~90 percent of mice from leukemia induced by both CSF3R-proximal and compound-mutant (**Figure 5A-F**). We could not detect minimal residual disease (MRD) determined by examining the venus expressing cells from the PB (**Figure 5A and D**).

To confirm that the treated mice were cured, secondary bone marrow transplants were performed using whole bone marrow cells from the vehicle and drug-treated mice (**Supplementary Figure 5**). Mice recipients of vehicle, BCI, and Trametinib treated primary bone marrow cells exhibited aggressive leukemic development with shorter disease latency (six weeks for $CSF3R^{T618I}$ and three weeks for $CSF3R^{T618I/Q741*}$) compared to primary transplanted mice (15 weeks for $CSF3R^{T618I}$ and 4-5 weeks for $CSF3R^{T618I/Q741*}$), **Supplementary Figure 5**. As expected, mice recipients of BCI+Trametinib treated primary bone marrow cells did not show any sign of leukemia and MRD cells determined by enumerating the venus positive cells by FACS (**Supplementary Figure 5**). Altogether, these data validate DUSP1dependence in CSF3R-induced leukemia and support developing a DUSP1-selective inhibitor for effective treatment outcomes.

Discussion

Both chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML) pose significant clinical challenge due to poor prognosis[21]. A vast majority of CNL patients harbor mutations in CSF3R, activating both JAK-STAT and MAPK signaling pathways[8,9,21,22] . Conversely, mutations activating the RAS-MAPK pathway are seemingly more common in aCML[23,24]. The efficacy of Jak2 inhibitor treatment were observed primarily with the CSF3R-proximal mutant ($CSF3R^{T618I}$), while the CSF3R compound mutation ($CSF3RT^{618I/Q741*)}$, associated with more aggressive leukemia, proved refractory to Ruxolitinib treatment[9,22,25].

Recent genomic and proteomic studies have revealed that, regardless of the genetic mutations they harbor, both CNL and aCML are fueled by enhanced MAPK signaling[9,10,12,26].

Consequently, the efficacy of Trametinib was noted in leukemic patients and preclinical mouse models of CNL and aCML[9,27]. As noted in the preclinical model, treatment with MAPK or JAK-STAT inhibitors suppresses leukemic progression but failed to induce a durable response[9]. Both Trametinib and Ruxolitinib exert cytostatic response and are rarely selective to leukemic clones. As a result, treatment outcomes to cytostatic drugs are short-lived and loss of therapeutic response and emergence of resistance is inevitable. Therefore, treatment strategies targeting leukemic clones are needed for durable and curative response. Herein, we show that DUSP1 (Dual specificity phosphatase 1) confers oncogene dependence in CSF3R-induced leukemia. Both genetic and pharmacological inhibition of DUSP1 selectively eradicated the leukemic cells and cured the mice of leukemia. Given that DUSP1 is not required for normal development, therapeutic targeting of DUSP1 in CNL/aCML would impart a curative response.

Deregulated ERK activity is commonly observed in many malignancies. Its oncogenic potential is governed by modulating the apoptotic-threshold mainly by stabilizing and/or activating the anti-apoptotic proteins, such as BCL-2 family members, and repressing the proapoptotic proteins, such as Bad and BIM[3,28,29]. Paradoxically, sustained ERK activity also induces robust apoptotic response which will be counter-selective for cellular transformation[2,30-32]. Therefore, most tumors exploit the MAPK negative regulators (e.g. PP2A and DUSP family members[12]) to fine-tune the signaling output to suppress the ERK induced apoptotic signaling while selectively promoting survival and proliferation[6]. We observed elevated but variable expression of Dusp family members (DUSP1, 2, 4, 5, 8, 9, 10,13,14, and 22) CSF3R expressing primary cells. Among these only DUSP1 was consistently induced in leukemic cells. Expression of other Dusp members varied with different CSF3R mutants and half of them were noted to be induced in CSF3R-wildtype cells suggesting that they may be least likely to be engaged in leukemogenesis. Genetic deletion of *Dusp1* selectively eradicated the leukemic cells further lends support to the notion that other Dusp family members are dispensable for CSF3R-induced leukemogenesis. Biochemical analysis revealed that DUSP1 selectively dampens the JNK1/2 driven apoptotic response to support leukemogenesis. Perhaps more interestingly, that DUSP1-regulated inhibition of apoptotic response appears to vary depending on the specific oncogenic drivers. For instance, in the context of BCR-ABL and JAK2^{V617F}-driven myeloproliferative neoplasms (MPN), suppression of apoptosis is mediated through the blockade of P38 activity[14,33], rather than JNK as noted in the context of CSF3R mutants. These observations suggest an oncogene-driven assembly of signaling complexes, leading to altered substrate selectivity in the apoptotic pathway[14,33]. These findings imply an oncogene-driven orchestration of signaling complexes unique to each oncogene, resulting in altered substrate selectivity within the apoptotic pathway. Future studies will elucidate the underlying mechanisms whether the altered substrate selectivity is due to spatial regulation of DUSP1, possibly modulated by scaffolding proteins, or it is caused by distinct signaling complexes uniquely orchestrated by each oncogene.

Chemical inhibition of DUSP1 by BCI failed to recapitulate genetic targeting of DUSP1. Because BCI inhibits both DUSP1 and DUSP6[14], concomitant inhibition of DUSP6 resulted in loss of inhibitory control on ERK1/2. Consequently, activated ERK suppressed the JNK activity, which we see when DUSP6 is inhibited by BCI. ERK mediated JNK inactivation has been reported in several cancer models, although the underlying mechanism is unclear[34]. Nonetheless, activation of other DUSP family members and Akt have been implicated[35]. Ectopic expression of DUSP6 in CSF3R mutant cells suppressed the pERK1/2 levels and restored BCI sensitivity. Thus, providing a direct evidence that the inefficacy of BCI was due to off-target inhibition of DUSP6 mediated by activated ERK1/2. Furthermore, leukemic mice treated with a combination of BCI and Trametinib suppressed the pERK1/2 and restored the JNK1/2 modulated apoptotic response and selective eradication of leukemic clones, which provides additional evidence that

the inefficacy of BCI treatment alone was due to ERK1/2 activation due to off-target inhibition of DUSP6. Mechanistically, BCI treatment depleted the level of BIM likely due to ERK1/2 mediated phosphorylation induced degradation[17,36-38]. In support, we show that inhibition of pERK1/2 by Trametinib stabilized the level of BIM. In contrast, JNK phosphorylated BIM follows a different fate where it has been shown to activate BAK and BAX or neutralize BCL2 resulting in a robust apoptotic response[39,40]. Perhaps more importantly, stabilization and transcriptional activation of P53 by activated JNK seemingly provides additional support to apoptotic response. For instance, phosphorylated P53 at Thr81 by JNK promotes its dimerization with P73, which induces the expression of several pro-apoptotic target genes, such as *Puma* and *Bax* [41]. Together our study demonstrated that the dynamic balance between ERK and JNK activation determines whether the cell survives or undergoes to apoptosis. MAPK negative regulator, DUSP1, regulates this balance to support the leukemogenesis induced by CSF3R mutants.

In conclusion, we show that DUSP1 confers oncogene dependence in CSF3R-induced leukemia. Deletion of *Dusp1* selectively induces JNK1/2 activity that promotes apoptosis by stabilizing and inducing the expression of P53 and BIM while downregulating the anti-apoptotic protein BCL2. In contrast, ERK1/2 negative regulator, DUSP6, functions as a tumor suppressor in CSF3R-induced leukemia that seemingly suppresses the elevated pERK1/2. Altogether these data provide evidence that the inhibitors selectively targeting DUSP1 would exert a durable or curative response in CNL/aCML. Finally, our studies expose a new Achilles heel to malignancies fueled by enhanced MAPK signaling and support for selective targeting of MAPK negative regulators for effective treatment outcomes.

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Author Contribution: M.K, M.A., and Z.K performed the experiments and analyzed the data. M.K., and M.A designed all experiments. M.K, and M.A wrote the manuscript.

Competing interests: No conflict of interest

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Figure legends:

Figure 1. Induced expression of *Dusp1* in CSF3R mutant expressing cells.
A. Heat map showing deregulated expression of MAPK pathway genes in BM derived Kit+ cells expressing different CSF3R mutants. Total RNA from the venus positive Kit cells after 24 hrs of transduction were subjected to RNA-seq analysis[9]. Total RNA from the vector (pMSCV-Ires-venus) transduced Kit+ cells was used to filter out differentially expressing cells. Differences in the levels of expression were measured using t-test between the samples from the leukemic variants ($CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*)}$ and non-leukemic truncation mutants ($CSF3R^{Q741*}$ and $CSF3R^{W791*}$). **B**. A bar graph showing the relative expression of *Dusp1* in LSK cells (Lin^-, Sca1+ and Kit+) expressing CSF3R mutants. **C**. Immunoblots from the total protein extracts of control (*sc-shRNA*) and *Dusp1* depleted BaF3 cells expressing CSF3R mutants grown without IL3 showing reduced p-ERK1/2 and induced activation of p-JNK1/2 upon *Dusp1* knock-down in cell expressing leukemic CSF3R mutants ($CSF3R^{T618I}$, $CSF3R^{T618I/Q741*,}$ and $CSF3R^{T618I/W791}$) . In contrast, the non-leukemic CSF3R truncation mutations exhibit modest elevation in p-ERK1/2 without any alteration in p-JNK1/2 levels. Expression levels were quantified and normalized to the control condition (pMIV-Vector expressing Sc-ShRNA normalized to to β-ACTIN). The resulting normalized values are presented below each blot for reference. BaF3 cells expressing *Dusp1-shRNA* resulting to ~6-70% knockdown at protein level and control *sc-shRNA* has been described earlier[14]. **D**. A cell proliferation growth curve of *Dusp1* depleted BaF3 cells expressing CSF3R mutants showing normal growth when grown with IL3. **E.** Growth curve showing significantly reduced proliferation upon *Dusp1* depletion in the absence of IL3. This assay revealed that the transformation potential of CSF3R is compromised upon *Dusp1* depletion. Presented data are from two independent experiments shown as the means ± SD. **p*<0.05, ***p*<0.01 and ****p*<0.001.

Figure 2. Deletion of *Dusp1* is synthetically lethal to CSF3R-induced leukemia.
A. Experimental design for evaluating the role of *Dusp1* in CNL/aCML. **B.** Percent CFUs from the C57Bl/6-WT and $Dusp1^{-/-}$ Kit^+ cells expressing leukemic $CSF3R^{T618I}$ (proximal) and $CSF3R^{T618I/Q741*}$(compound mutation). The data shown are the mean colony number from two independent experiments ± S.D. **C-E.** Shown are the leukemia development in mice transplanted with wild-type BM-derived Kit+ cells expressing $CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*}$. **C.** Peripheral blood smear (top panel) and WBC levels determined biweekly (bottom panel). **D.** Survival curve of leukemic mice transplanted with $CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*}$ expressing Kit^+ cells. **E.** Shown is the $venus^+$ cells as a surrogate leukemic burden from the peripheral blood. Dotted lines represent normal WBC levels. Representative data are from the two independent transplant experiments. **F-H**. Mice transplanted with *Dusp1* deficient BM-derived Kit+ cells expressing $CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*}$ are gradually eradicated from the BM. **F.** Peripheral blood smear (top panel) and WBC levels determined biweekly (bottom panel) do not show any elevation of WBC levels. **G.** Survival curve showing prolong survival of mice transplanted with *Dusp1* deficient Kit+ cells expressing $CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*}$. **H.** Kit cells expressing $CSF3R^{T618I}$ and $CSF3R^{T618I/Q741*}$ are progressively removed from the peripheral blood while maintaining the vector expressing cells. Representative data are from two independent experiments (five mice per group) shown as the means ± SD. **p*<0.05, ***p*<0.01 and ****p*<0.001.

Figure 3. Chemical inhibition of DUSP1 by BCI is ineffective.
A-C. Mice transplanted with wild-type Kit+ cells expressing CSF3R^{T618I}. Graphs showing the total WBC levels (A), survival (B) and percent venus positive cells as a surrogate leukemic burden (C). **D-F.** Leukemic progression in mice transplanted with CSF3R$^{T618I/Q741*}$ expressing Kit^{+} cells. Graphs showing the total WBC levels (D), survival (E) and percent venus positive cells as a surrogate leukemic burden (F). Dotted lines represent normal WBC levels. Treatment with BCI alone or with Ruxolitinib is ineffective in suppressing the disease in both models of CSF3R induced leukemia. Representative data are from two independent experiments (three mice per group) shown as the means ± SD. *p<0.05, **p<0.01 and ***p<0.001.

Figure 4. Activation of p-ERK1/2 due to off-target inhibition of DUSP6 by BCI abrogated its antileukemic response.
A. A model depicting JNK1/2 and P38 activation in leukemic cells upon *Dusp1* knock-down. **B.** Consequently, expression of pro-apoptotic proteins BIM and P53 are induced while expression of anti-apoptotic protein BCL2 was significantly reduced. Expression levels were quantified and normalized to the control condition (pMIV-Vector expressing Sc-ShRNA normalized to β-ACTIN). The resulting normalized values are presented below each blot for reference. **C.** A model depicting chemical inhibition of DUSP1 and DUSP6 by BCI activated p-ERK1/2 that suppressed JNK1/2 mediated apoptotic response. **D**. Immunoblots showing the activation of p-ERK1/2 upon BCI treatment resulting to inhibition of JNK1/2 and reduced expression of BIM while the levels of P53 and BCL2 were unaffected. Treatment with Trametinib alone restored JNK1/2 activation and the level of BIM with modest reduction on BCL2 levels. Interestingly, cells treated with BCI+Tram exhibit induced expression of P53 with reduced BCL2 levels. Representative blots are from two independent experiments. Expression levels were assessed and normalized to control condition (vehicle treatment normalized to β-ACTIN). The resulting normalized values are indicated below each blot.

Figure 5. BCI in combination with Trametinib eradicated the leukemic clones and cured the leukemic mice.
A-C. Mice transplanted with wild-type Kit^{+} cells expressing CSF3R^{T618I} treated with BCI and Trametinib alone or in combination. Graphs showing the percent venus positive cells as a surrogate leukemic burden (A), total WBC levels (B), and survival (C). **D-F.** Mice transplanted with compound mutation treated with BCI and Trametinib alone or in combination. Graphs showing the percent venus positive cells as a surrogate leukemic burden (D), total WBC level (E), and survival (F). Treatment with Trametinib suppresses the leukemia but lacked clonal selectivity resulting to cytostatic response. Treatment with BCI lacked both anti leukemic response and clonal selectivity. However, a combination of Trametinib and BCI not only suppressed the WBC levels but also effectively eradicated the leukemic clones resulting to cure in both models of CSF3R induced leukemia. Representative data are from two independent experiments (three mice per group) shown as the means ± SD. *p<0.05, **p<0.01 and ***p<0.001.

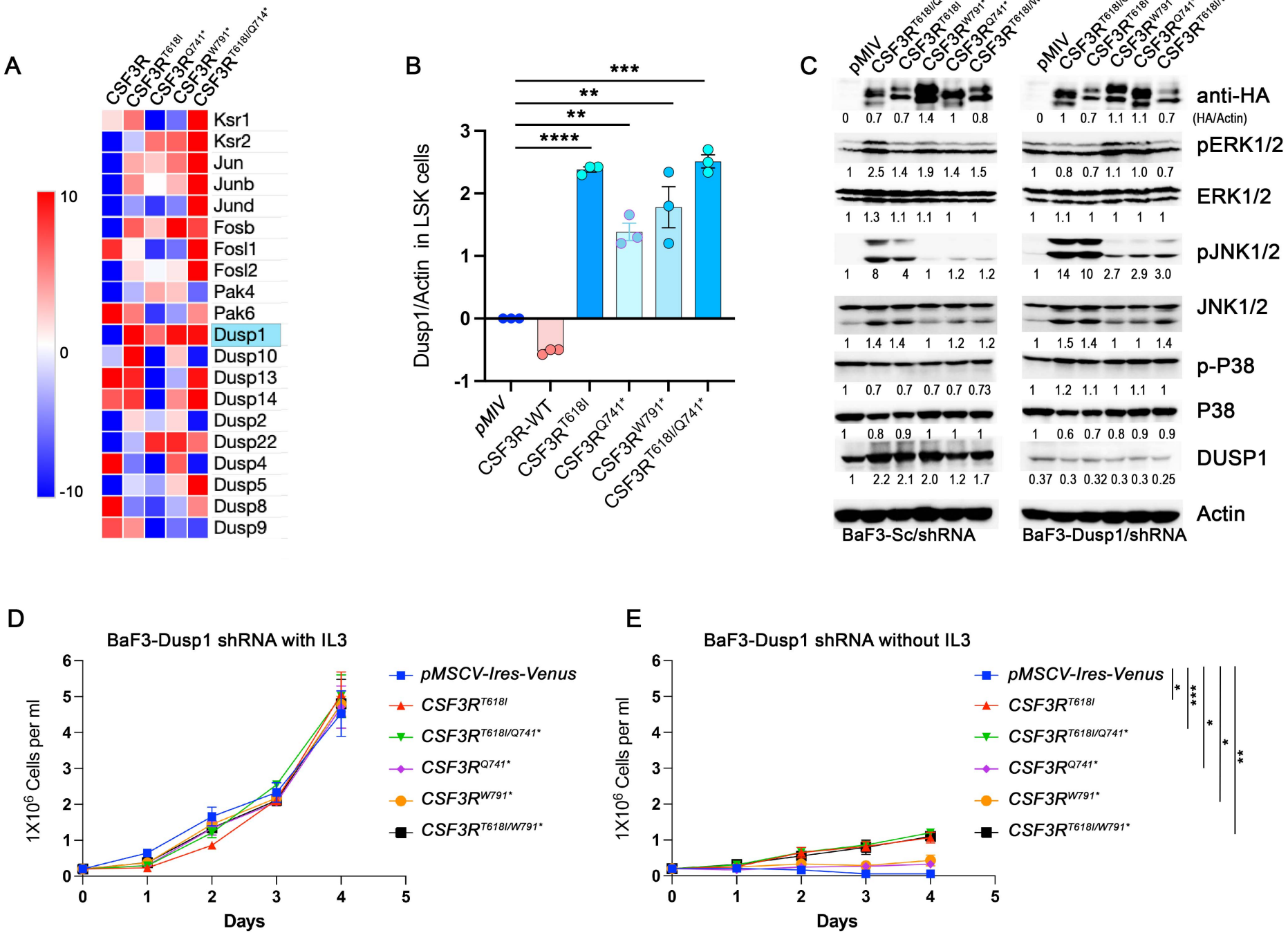

Figure 1

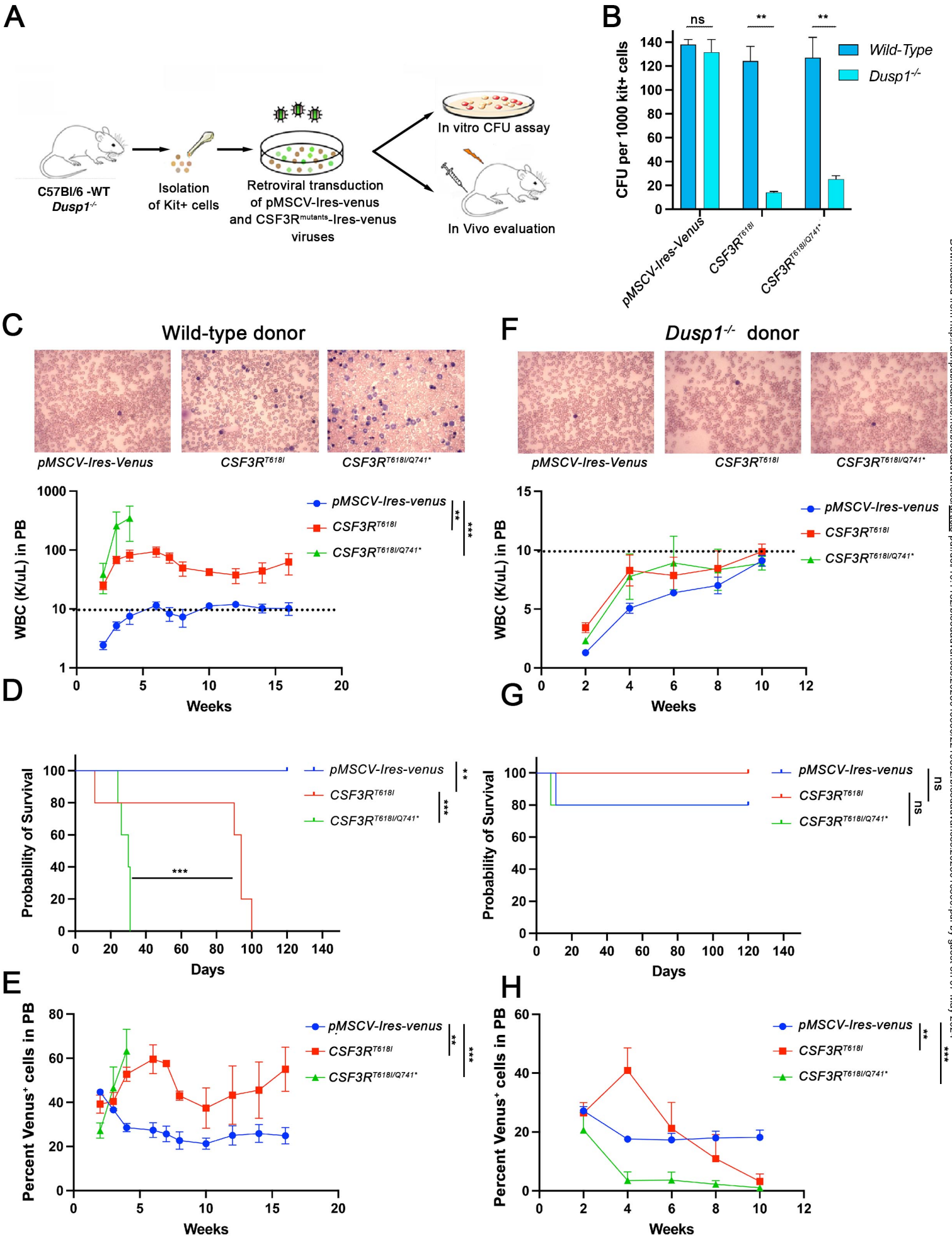

Figure 2

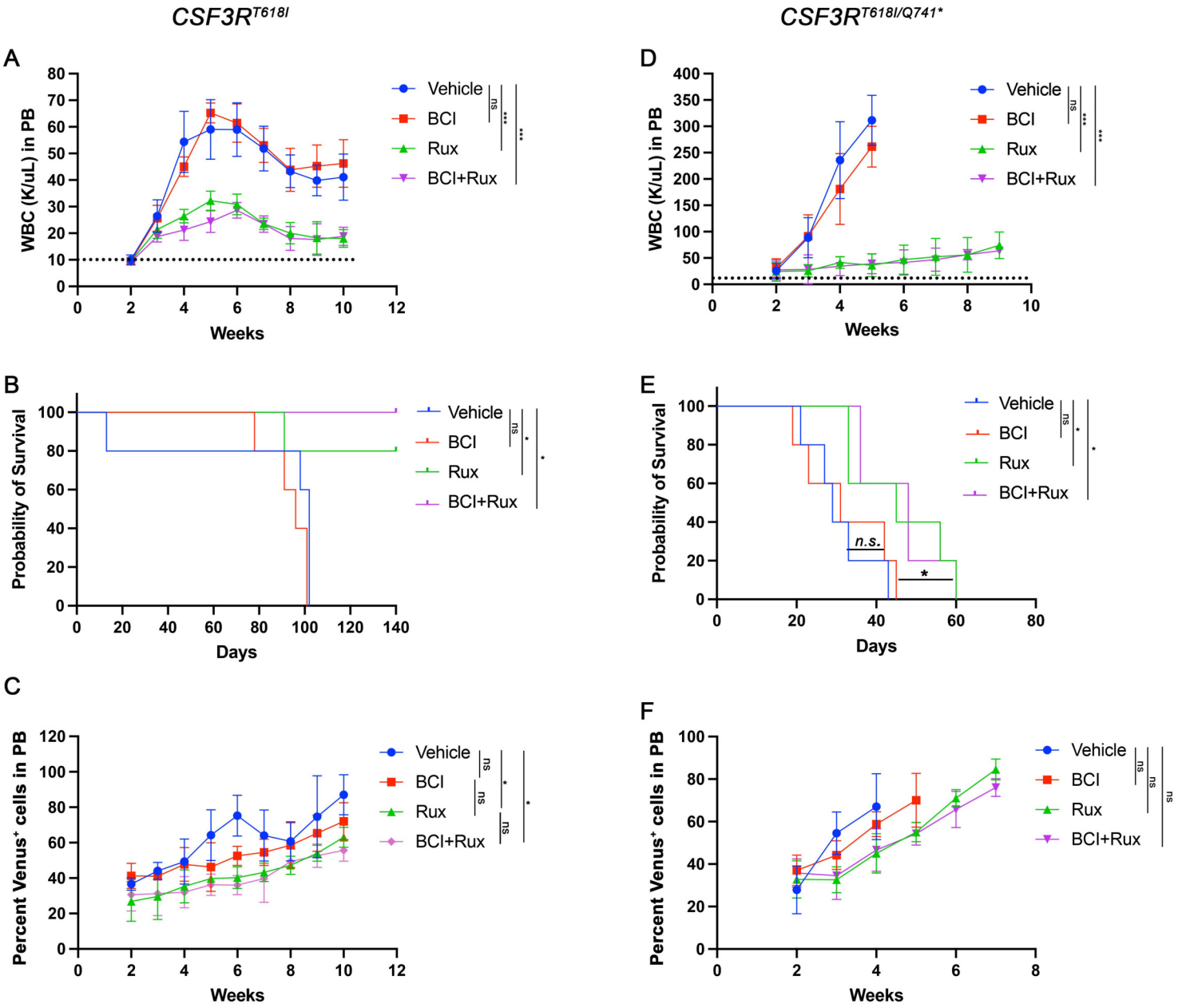

Figure 3

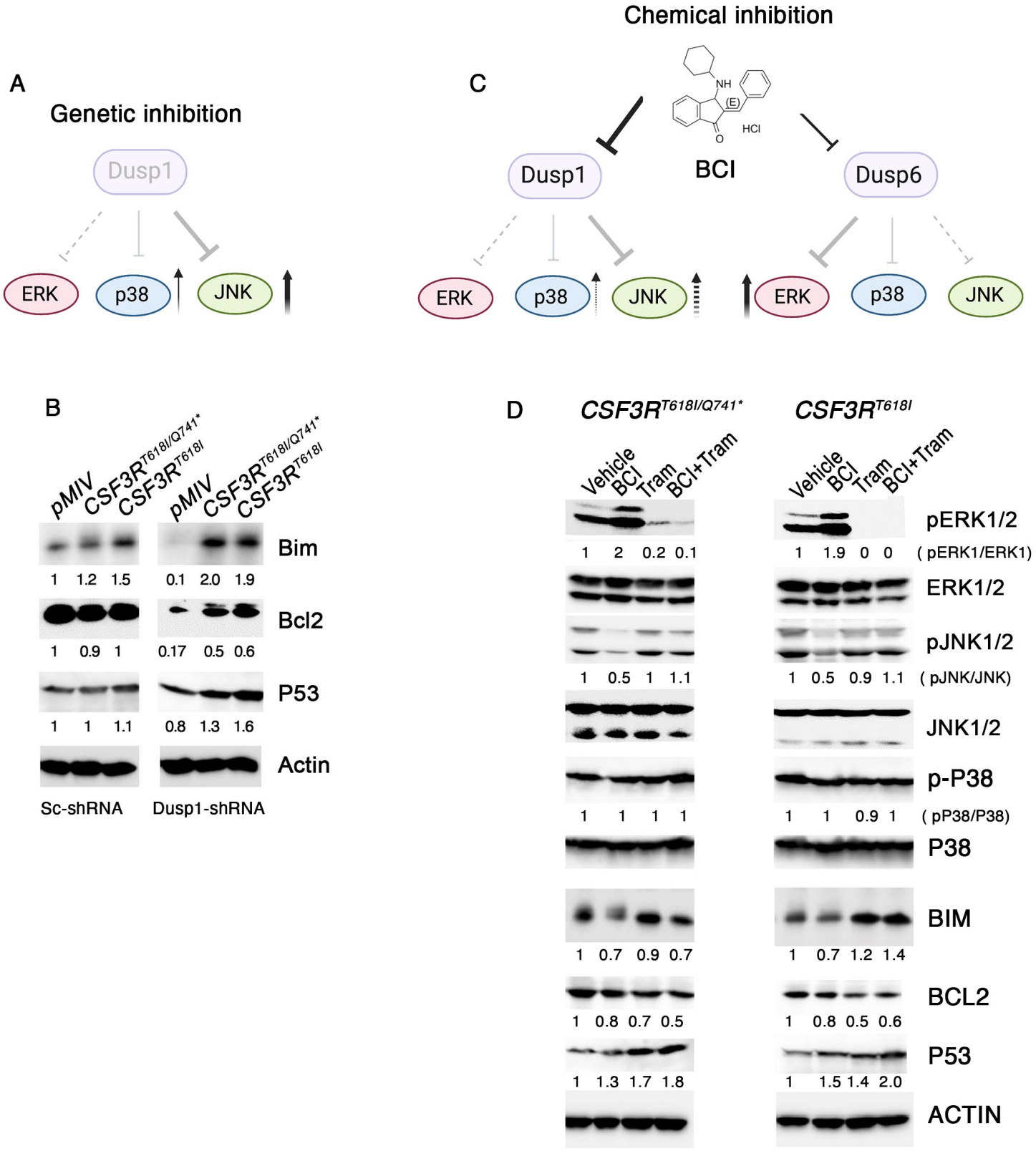

Figure 4

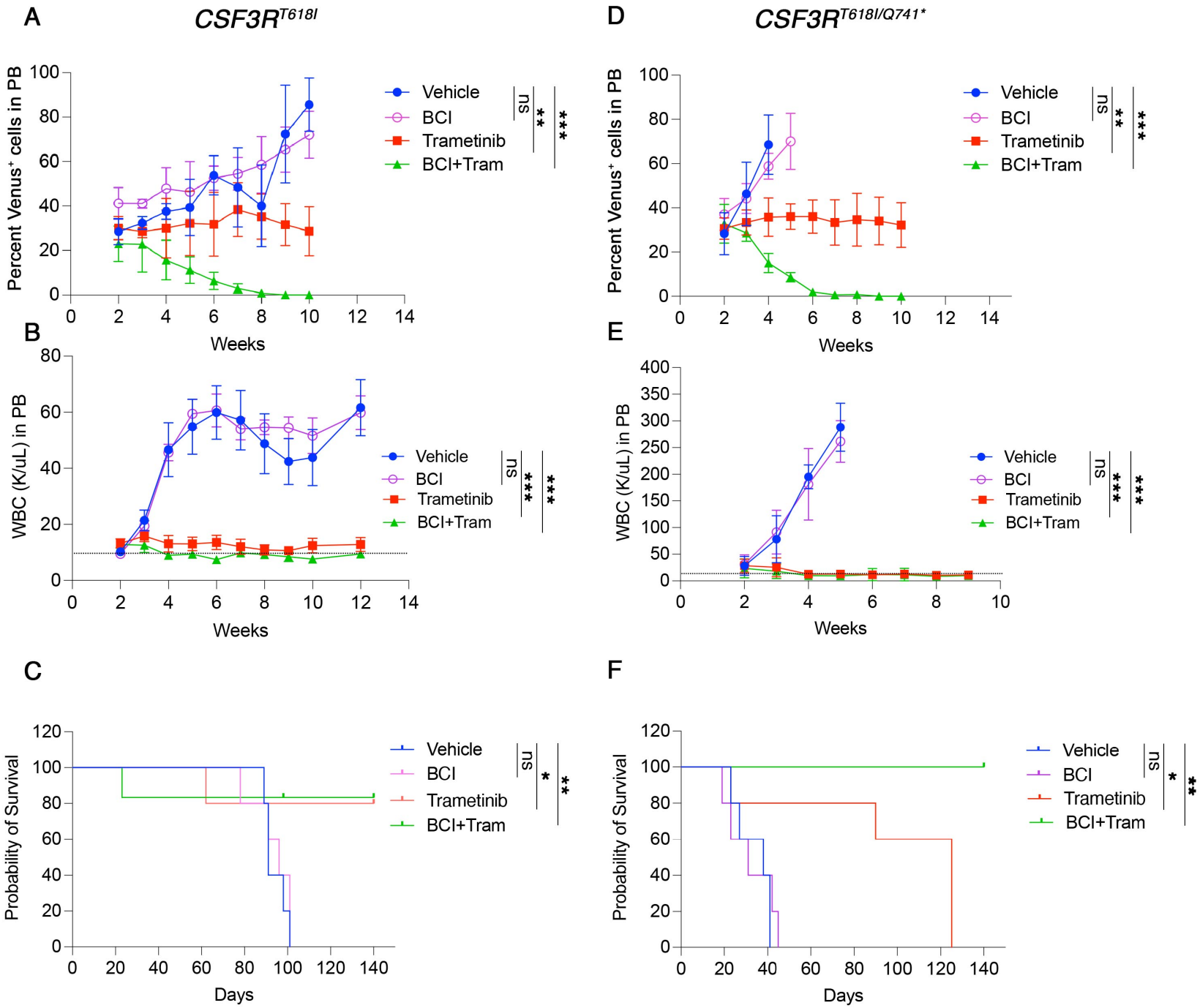

Figure 5