CXCR4 allows T cell acute lymphoblastic leukemia to escape from JAK1/2 and BCL2 inhibition through CNS infiltration

Short Title: JAK1/2-BCL2-CXCR4 inhibition for T-ALL treatment

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Abstract

Targeting the JAK/STAT and BCL2 pathways in patients with relapsed/refractory T cell acute lymphoblastic leukemia (T-ALL) may provide an alternative approach to achieve clinical remissions. Ruxolitinib and venetoclax show a dose-dependent effect on T-ALL individually, but combination treatment reduces survival and proliferation of T-ALL in vitro. Using a xenograft model, the combination treatment fails to improve survival, with death from hind limb paralysis. Despite on-target inhibition by the drugs, histopathology demonstrates increased leukemic infiltration into the central nervous system (CNS) as compared to liver or bone marrow. Liquid chromatography-tandem mass spectroscopy shows that ruxolitinib and venetoclax insufficiently cross into the CNS. The addition of the CXCR4 inhibitor plerixafor with ruxolitinib and venetoclax reduces clinical scores and enhances survival. While combination therapy with ruxolitinib and venetoclax shows promise for treating T-ALL, additional inhibition of the CXCR4-CXCL12 axis may be needed to maximize the possibility of complete remission.

Keywords

T cell acute lymphoblastic leukemia; ruxolitniib; venetoclax; plerixafor; JAK/STAT; BCL2

Introduction

T cell acute lymphoblastic leukemia (T-ALL) accounts for 25% of adult and 15% of pediatric acute lymphoblastic leukemia (ALL) cases. T-ALL occurs when mutated thymocytes that are destined for cell death survive and acquire additional mutations that promote full malignant transformation[1]. Cure rates, respectively, reach 60% in adults and over 80% in pediatric patients when treated with combination chemotherapy protocols[2-4]. However, resistance to first-line therapy is seen in 25% of children and more than 50% of adults, and the cohorts that do respond to current ALL chemotherapy protocols often suffer from both acute and chronic toxic side-effects despite treatment success.[5] Therefore, there is a need for the development of novel, non-chemotherapeutic treatments for this type of blood cancer[6,7].

In order to improve prognosis and mitigate side-effects seen with current chemotherapy treatments, it is crucial to evaluate the underlying molecular pathways that drive T-ALL and contribute to chemotherapy resistance. Over the last decade, the Janus Activating Kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) pathway has been to shown to play a critical pathogenic role in several hematologic malignancies including T-ALL[8,9]. Activation of JAK tyrosine kinases leads to the subsequent phosphorylation and activation of STAT transcription factors[10-12]. Additionally, many T-ALL express elevated levels of the pro-survival protein BCL2[13-15][6]. These pathways allow for increased T-ALL proliferation through the JAK/STAT pathway and increased T-ALL survival through augmented BCL2 expression.

Ruxolitinib is an FDA approved small molecule inhibitor that targets JAK1 and JAK2. Ruxolitinib prevents the tyrosine phosphorylation of STAT1/STAT3/STAT5, which are downstream of cytokine receptors that drive T-ALL proliferation (e.g. IL-7), and should function to inhibit the proliferative properties seen in T-ALL. Unfortunately for most patients, chronic therapy with ruxolitinib results in reactivation of JAK/STAT signaling and prevents T-ALL remission[16]. In

these cases, BCL2 may also be hijacked and upregulated to promote survival of these cancer cells.

Venetoclax is a highly potent and specific BCL2 mimetic that acts as an artificial sensitizer, binding to the BH3 groove of the BCL2 protein without targeting platelets. This causes the downregulation of BCL2 and eventual cell death of T-ALL[17-19]. The combination of ruxolitinib and venetoclax is emerging as a viable treatment option for relapsed disease. These drugs have been utilized previously to treat IL-7 receptor alpha (IL7Rα)-mutated T-ALL, which prolongs survival in a preclinical model but ultimately all animals succumb to leukemia[20]. Because the central nervous system (CNS) is a common site of T-ALL involvement, [2,21] [2,21] [2,21] [2,21] [2,21] [2,21] [2,21] [2,21] one potential limitation of inhibiting these pathways *in vivo* is that it is unknown if these drugs can cross the blood-brain barrier (BBB)[2,21]. Also, the mechanism by which T-ALL would escape the combination of ruxolitinib and venetoclax is entirely unknown.

The CXCR4-CXCL12 axis has been implicated as a potential pathway that drives T-ALL invasion into the CNS[22-25]. This study will demonstrate that ruxolitinib and venetoclax are efficacious *in vitro* to treat T-ALL, but are not effective *in vivo* by showing for the first time their inability to effectively cross the blood-brain-barrier (BBB) and treat T-ALL in the CNS. By deleting the CXCR4 gene from T-ALL or blocking its activity with plerixafor, prolonged survival was observed *in vivo* with decreased total and neurologic clinical scores. Thus, in the setting of CXCR4 inhibition, T-ALL CNS infiltration can be blocked and allow for the systemic clearance of T-ALL by ruxolitinib and venetoclax.

Methods

Tumor cell lines:

Jurkat (TIB-152) clone E6-1 and Loucy (CRL-2629) (ATCC Manassas, VA) were used as human T-ALL cell lines. Jurkat-GFP cell line was generated by transfecting $2x10^5$ Jurkat cells using the Lonza 4D-Nucleofector X Unit (Basel, Switzerland). The Nucleofector was used to deliver 1µg of px330 plasmid encoding for Cas9 protein and a gRNA for the AAVS1 locus, as well as 1µg of an AAVS1-CAGGS-EGFP plasmid to serve as the donor template. The Nucleofection was carried out in SE buffer and the pulse code for the transfection was CL-120. The cells were cultured in RPMI post nucleofection and sorted on a FACSAria for GFP^+ cells. Cells were cultured in RPMI 1640 1x medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini bio-products, Sacramento, CA), L-Glutamine (2 mM), penicillin/streptomycin (100 µ/ml) at 37°C, 5% CO_2 and 95% humidity. Excluding FBS, all other media ingredients were purchased from Corning, Corning, NY. All cell lines were mycoplasma tested and authenticated prior to use (IDEXX Bioresearch, Westbrook, ME).

In vivo T-ALL xenograft model:

NOD/SCID/*Il2rg*^{tm1wjl}/Szj (NSG) breeder mice were purchased from Jackson Labs (Bar Harbor, ME) and bred internally at the Biomedical Research Model Services Breeding Core at University of Wisconsin-Madison. Mice were housed in accordance with the Guide for the Care and Use of Laboratory Mice and experiments were performed under an IACUC approved animal protocol. Both male and female mice aged 8-16 weeks were randomized into control or treatment groups. On Day +0, $2x10^6$ Jurkat cells in 200 µl of 1x PBS were injected intravenously into NSG mice to generate a xenograft T-ALL mouse model. Using flow cytometric analysis, at least 1% anti-human CD45 cancer burden was documented in the peripheral blood (usually day 5-7) before initiating treatments. Mice were orally gavaged once daily for 14-days with vehicle(s), 100µl 30 mg/kg/day ruxolitinib, 100µl 35 mg/kg/day venetoclax, or combination. Mice were monitored for survival, % weight change, and clinical symptoms of disease (activity, hunch, and hind-limb paralysis) using a modified scoring system (Supplemental Table 1).

Ruxolitinib and venetoclax treatments:

Ruxolitinib (INCB018424) and venetoclax (ABT-199) were purchased in powdered form (Active Biochemicals, Kowloon Bay, Kowloon, Hong Kong, Cat # A-1134, A-1231). For *in vitro* use, ruxolitinib was resuspended in 100% DMSO at a concentration of 100 mg/ml and diluted to a working stock of 50 μM (final concentration of DMSO being 10%). For *in vivo* use, ruxolitinib in 100% DMSO was added to 5% N,N-dimethylacetamide (Cat # D137510-500ml, Sigma Aldrich, St. Louis, MO) + 0.5% methylcellulose (Cat # ME136, Spectrum Chemical, New Brunswick, NJ) in H_2O. For *in vitro* use, venetoclax was resuspended in 100% warm DMSO at a concentration of 50 mg/ml and diluted to a working stock of 200 nM (final concentration of DMSO being 11%). For *in vivo* use, venetoclax was resuspended in 100% EtOH at a concentration of 100 mg/ml. Appropriate amount of venetoclax was resuspended in 10% -100% EtOH + 30% PEG 400 (Cat # 1008415, Rigaku, The Woodlands, TX) + 60% Phosal 50 (Lipoid, Ludwigshafen, Germany).

For other assays, please see Supplementary Methods.

Statistical methods:

Statistics were performed using GraphPad Prism version 7.0 for the Macintosh OS (GraphPad Software, San Diego, CA). Data were expressed as mean ± SEM. For analysis of three or more groups, a non-parametric ANOVA test was performed with the Bonferroni or Sidak's multiple comparisons post-test. Analysis of differences between two normally distributed test groups was performed using a two-sided Mann Whitney test. A p-value less than 0.05 was considered statistically significant.

Results

JAK/STAT and BCL2 are dysregulated in T-ALL

To confirm that both STATs and BCL2 are dysregulated in T-ALL, we first performed flow cytometric analysis of two T-ALL cell lines, Jurkat and Loucy, and compared their expression of pSTAT1, pSTAT3 and BCL2 against two healthy controls. The T-ALL lines exhibited a complete dysregulation of pSTAT expression with decreased levels of pSTAT1 and increased levels of pSTAT3 compared to healthy controls (Figure 1A-B). We did not detect any expression of pSTAT5 in either T-ALL line or healthy control (data not shown). Additionally, BCL2 was upregulated in the T-ALL lines (Figure 1C). We next did a secondary analysis of RNA-Seq data from a Children's Oncology Group study of T-ALL patients[26] and analyzed STAT1, STAT3 and BCL2 expression upon initial diagnosis or relapse. We observed that STAT expression remained constant even after relapse, while the expression of BCL2 decreased, suggesting that these pathways are important for T-ALL survival (Figure 1D-F).

Single agent and combination inhibitor responses of ruxolitinib and venetoclax decrease the survival and proliferation of T-ALL cells *in vitro*

To investigate the relevance of the JAK/STAT and BCL2 pathways on T-ALL proliferation and cell survival, Jurkat (a mature T-ALL) and Loucy (an early precursor T-ALL) were assessed following treatment with either ruxolitinib or venetoclax.[15,27] Both of these cell lines were treated with a serial dose of ruxolitinib and venetoclax over the course of 72 h and evaluated using a trypan blue exclusion assay and MTT proliferation assay. Both ruxolitinib or venetoclax were able to decrease the survival and proliferation of both Jurkat and Loucy cell lines after 24, 48, and 72 h of treatment in a dose-dependent manner (Figure 2 and Supplemental Figure 1-2).

After determining that ruxolitinib and venetoclax individually affect survival and proliferation of T-ALL, T-ALL cells were treated with ruxolitinib and venetoclax in combination and evaluated again for survival and proliferation. This combination dose was then tested for survival and proliferation in comparison to single-dose and DMSO controls in both Jurkat and Loucy cell lines (Figure 3A-B, Supplemental Figure 3). We also assessed both Annexin V and 7-AAD via flow cytometry and found increased Annexin V and 7-AAD in both the Jurkat and Loucy cell lines (Figure 3C). Lastly, the Jurkat cell line also showed a reduction in survival for up to 5 days in culture utilizing Incucyte live-cell imaging technology ($p < 0.0001$ compared to no treatment) (Figure 3D).

Ruxolitinib and venetoclax fail to treat T-ALL *in vivo* due to leukemia CNS infiltration

Based on the efficacy of ruxolitinib and venetoclax observed *in vitro*, a xenograft model of T-ALL was established utilizing Jurkat cells and NSG mice[28,29] to test these drugs as a combination therapy *in vivo*. While single agent studies have used venetoclax doses up to 100mg/kg/day[12], we observed that these doses were not well tolerated when given in combination with 30 mg/kg/day ruxolitinib causing lethal gastrointestinal toxicity in NSG mice minutes to hours after administration. However, we did find that 35 mg/kg/day venetoclax was able to be tolerated, and so was used in our combination treatment groups with 30mg/kg/day ruxolitinib (and as a single agent dose as a control) for 14 days after cancer burden establishment. No diarrhea or other gastrointestinal toxicity was observed. *In vivo* dosing revealed optimal doses of 30 mg/kg/day of ruxolitinib and 35 mg/kg/day of venetoclax delivered via oral gavage once daily[15,17,30,31] for 14 days after cancer burden establishment (Figure 4A). Surprisingly, ruxolitinib and venetoclax failed to improve clinical scores (Figure 4B) or survival (Figure 4C) after T-ALL challenge when given as single agents or in combination with the cause of death ultimately due to hind limb paralysis (Supplemental Figure 4), suggesting using reduced doses to limit toxicity may have reduced efficacy.

We next injected NSG mice with GFP expressing Jurkat cells and analyzed the cancer burden (without ruxolitinib or venetoclax treatment) by flow cytometry for an anti-human $CD45^{+}GFP^{+}$ double population in the bone marrow, spleen, brain, and spinal cord over the course of the disease (approximately 50-60 days). Interestingly, the T-ALL burden was primarily in the spinal cord and eventually the brain, but not the bone marrow and spleen (Figure 5A-C). While both inhibitors are FDA approved and currently used in the clinic, there is no published data that shows if either inhibitor has the ability to effectively penetrate the CNS and treat T-ALL. Naïve mice were treated by oral gavage with the highest published *in vivo* single dose of either ruxolitinib (50 mg/kg)[30] or venetoclax (100 mg/kg),[15] and were then sacrificed at 3 hours post-ruxolitinib or 16 hours post-venetoclax[32,33]. The peripheral blood, spinal cord, and brain were then analyzed for the presence of either inhibitor by LC-MS-MS. We observed that there was a 5X and 100X reduction in the amount of ruxolitinib and venetoclax respectively in the spinal cord/brain compared to the serum (Figure 5D). These data show that when administered *in vivo* at doses of 30 mg/kg/day ruxolitinib and 35 mg/kg/day venetoclax, these drugs insufficiently penetrate the BBB, potentially explaining why they fail to treat CNS T-ALL disease burden in the Jurkat *in vivo* model.

The CXCR4/CXCL12 pathway is upregulated in Jurkat cells and mediates CNS penetration.

To determine how Jurkat cells were infiltrating the CNS, we first examined the CXCR4 expression on Jurkat cells compared to healthy human controls. The chemokine CXCR4 recognizes CXCL12 or stromal-derived factor 1 (SDF-1) that directs cells to the CNS. We found that Jurkat cells has significantly elevated levels of CXCR4 compared to healthy human $CD3^{+}$ cells (Figure 6A).

Using CRISPR-Cas9 technology we deleted the CXCR4 gene in Jurkat cells (Figure 6B). We then injected the CXCR4-KO, or no-guide control (NGC) Jurkat cells, into mice and monitored them for clinical symptoms of T-ALL. The NGC group developed rapid symptoms of CNS disease and had to be euthanized by day 35 while the mice transplanted with CXCR4-KO cells survived until day 80-100 and only had a few mice develop signs of CNS pathology (Figure 6C-E). Lastly, in combination with ruxolitinib and venetoclax therapy, we used two different approaches to block CNS infiltration in vivo that are relevant to the clinic. The first approach attempted to block CXCR4 with an anti-CXCR4 antibody but no efficacy was detected (Supplemental Figure 5). The second approach used plerixafor, a small molecule inhibitor of the CXCR4/CXCL12 axis. The use of plerixafor in combination with ruxolitinib and venetoclax significantly reduced clinical scores (Figure 6F) and extended survival (Figure 6G) of mice compared to the condition without plerixafor. Overall, these data show that blocking T-ALL infiltration and persistence in the CNS with a CXCR4 small molecule inhibitor is a feasible strategy to prevent T-ALL escape from treatment with ruxolitinib and venetoclax.

Discussion:

Relapsed or refractory T-ALL remains a significant challenge to manage, especially since most patients are heavily pretreated with chemotherapy that causes significant end organ toxicity. Development of targeted therapies that inhibit pathways exploited by T-ALL to augment proliferation and survival may improve outcomes while also minimizing systemic toxicity. Prior studies involving RNA or whole genome sequencing of T-ALL samples identified the JAK/STAT pathway[26,34,35] and the BCL2 pathway as being dysregulated[15]. In this study ruxolitinib, a JAK1/2 inhibitor, showed efficacy against T-ALL *in vitro* as measured by viability and proliferation assays. We also found that venetoclax, a potent BCL2 inhibitor demonstrated the same effects *in vitro.* Furthermore, it was demonstrated that when employing these two inhibitors together *in vitro*, the combination therapy demonstrated an increased effect as

measured by increased apoptosis and a decrease in proliferation compared to either inhibitor alone. This data confirmed findings previously demonstrated in T-ALL[20,36,37] and other cancers.[38-40]

Ruxolitinib treatment alone has been considered a breakthrough therapy for myeloproliferative neoplasms but long-term studies have shown that this treatment is not curative and does not lead to molecular or pathological remission. This may be caused by the disease evading treatment and utilizing an alternative survival pathway, such as the BCL2 pathway. While Senkevitch et al 2018 demonstrated a robust synergistic effect of combined JAK/STAT and BCL2 inhibition *in vivo* in IL7Rα+ T-ALL, all mice succumbed to T-ALL[20]. Our study demonstrates for the first time that T-ALL progression after combined ruxolitinib and venetoclax treatment arises from CNS invasion mediated by CXCR4, a homing pathway previously identified in untreated T-ALL cells.[22] The xenograft model of T-ALL with Jurkat cells presents as CNS disease with the cancer burden homing primarily into the spinal cord, rather than a primary systemic form where the burden is primarily seen in the bone marrow, spleen, and liver.[41] This xenograft model has clinical significance given that CNS relapse is common for T-ALL, and is a strong driver for a poor prognosis.[42]

The current standard of treating CNS leukemia is either by administering repeated doses of intrathecal chemotherapy or giving irradiation to the neuro-axis. Identification of agents that are effective in the CNS when administered systemically is an urgent need. This study demonstrated for the first time that while ruxolitinib and venetoclax have activity *in vitro,* they may be ineffective *in vivo* since they are unable to effectively cross the BBB and target CNS leukemia. LC-MS-MS demonstrated that low amounts of ruxolitinib (administered at the highest published dose 50mg/kg)[30] can be detected in the spinal cord and brain of NSG mice and low amounts of venetoclax (administered at the highest published dose 100mg/kg[1,15]) can be

detected in the spinal cord, though they are not high enough concentrations to be expected to have an anti-tumor effect at a physiological dose (ruxolitinib 30mg/kg/day; venetoclax 35mg/kg/day). These data could be informative as future generations of these small molecule inhibitors may need to be engineered to allow for better CNS penetration.

Further we dissected the homing mechanism demonstrated with Jurkat cells in NSG mice. Several studies of human T-ALL have demonstrated that CNS relapsed T-ALL occurs in part through the CXCR4-CXCL12 chemokine pathway[22,24,25,43]. We confirmed that Jurkat cells utilize this pathway to escape treatment by ruxolitinib and venetoclax and infiltrate the CNS. CXCR4 is abundantly present on the surface of Jurkat cells and CXCL12 is highly abundant in the CNS. Since murine CXCL12 can bind the human CXCR4 receptor, human T-ALL can enter the CNS of NSG mice[44,45]. Utilizing CRISPR-Cas9 to delete CXCR4 from the surface of Jurkat cells, we determined that CXCR4 plays a critical role in Jurkat cell homing to not only the CNS but also non-CNS sites. CXCR4 deletion or inhibition in T-ALL cells significantly delays lethality by 60-70 days while reducing overall and neurological clinical scores, showing that CXCR4 plays a critical role in T-ALL progression. Interestingly though, the use of an anti-CXCR4 antibody failed to prevent CNS penetration. This is most likely due to the poor ability of antibodies to cross the BBB with one report suggesting antibodies exist at 1000X lower concentration in the CNS than in the serum[46]. Additionally, we treated our mice with anti-CXCR4 after Jurkat injection suggesting that Jurkat cells had already infiltrated the CNS and evaded anti-CXCR4 treatment. Since plerixafor was efficacious in our model, we hypothesize that plerixafor is capable of crossing the BBB, blocking CXCR4 signaling and mediating the egress of Jurkat cells out of the CNS.

Development of novel therapies for relapsed/refractory T-ALL is a high unmet need given the poor prognosis of this cancer in children and adults. Inhibiting the JAK/STAT pathway along with

BCL2, using ruxolitinib and venetoclax, is an effective treatment for relapsed T-ALL, but requires additional inhibition of CXCR4 to prevent CNS invasion and relapse after treatment. Future studies could consider targeting all three pathways as a means of inducing a sustained complete remission. Combination of ruxolitinib and venetoclax with cell-based therapies, like T or NK cells, could also be considered since immune effector cells can easily cross the BBB, but the impact of these drugs on these cell subsets will need to be explored carefully.

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Authorship Contributions:

K.L.W. and C.M.C designed the experiments, analyzed and interpreted results and wrote the manuscript; N.J.H, S.P.R., S.A.K, F.Z., S.L.O, M.M.C., A.E.Q., A.S.F., and T.B.G. conducted experiments and analyzed data; N.J.H, S.A.K. and S.L.O analyzed data and generated figures; and S.P.R., D.P.T., M.N.B. and L.R. discussed and interpreted results. All authors read and approved the manuscript.

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Figure Legends:

Figure 1. JAK/STAT and BCL2 pathways are dysregulated in T-ALL. (A-C) Histograms of T-ALL lines Jurkat and Loucy stained for pSTAT1 (A), pSTAT3 (B) or BCL2 (C) and compared to healthy controls and an isotype control antibody. Bar graphs represent the fold MFI (target MFI / isotype MFI) of two independent experiments (for cancer lines) and two healthy donors. (D-F)

Normalized FPKM values of STAT1 (D), STAT3 (E) and BCL2 (F) at diagnosis and relapse from a Children's Oncology Group T-ALL RNA-seq dataset[26].

Figure 2. Potency of single-agent ruxolitinib and venetoclax against T-ALL lines. Trypan blue (A-B) and MTT proliferation data (C-D) of serially diluted ruxolitinib (A, C) and venetoclax (B, D) at 48 hours. Two independent experiments were performed with triplicate measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

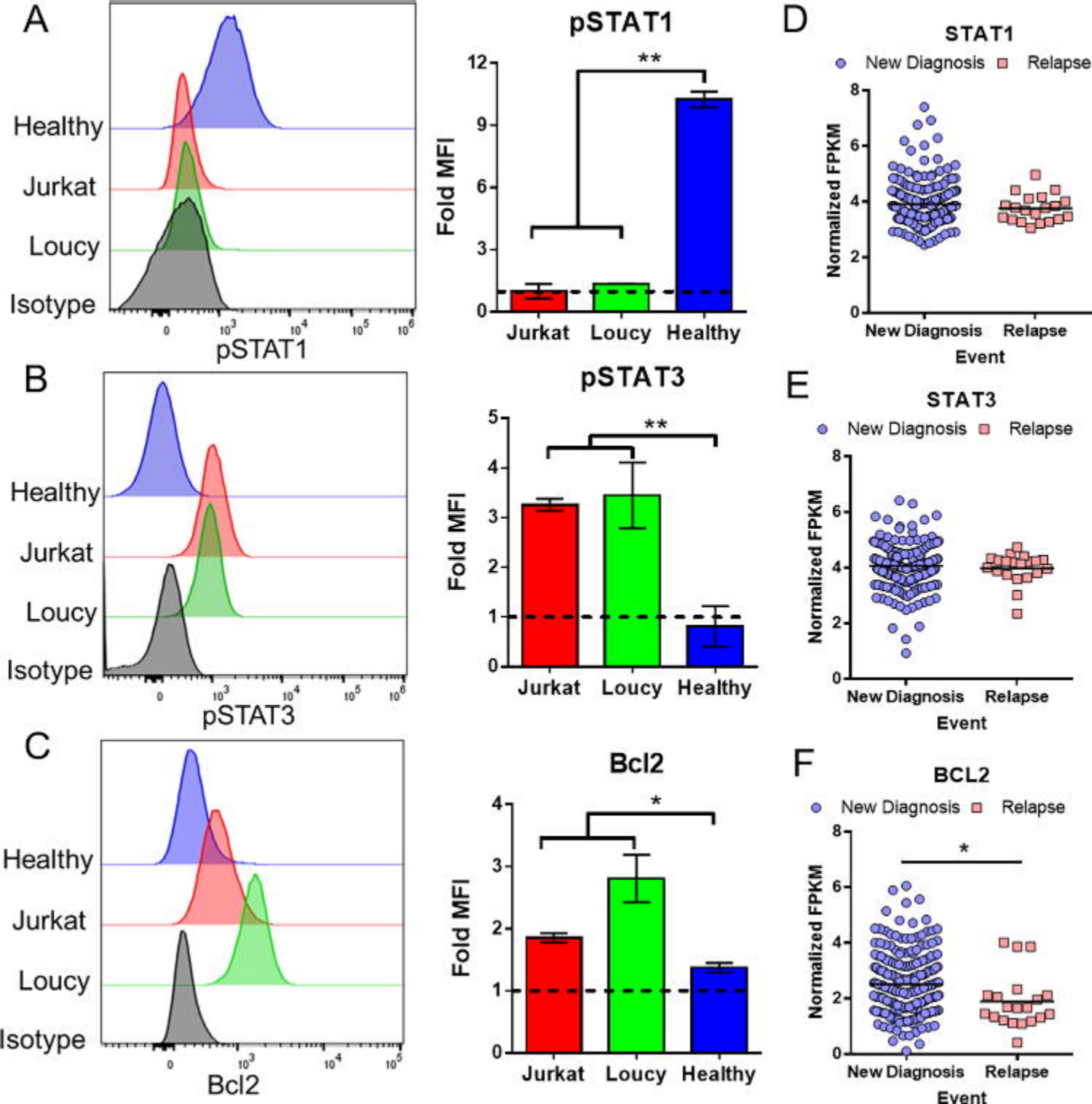
Figure 3. Ruxolitinib and venetoclax combination treatment is more potent than single-agent treatment. Trypan blue (A), MTT proliferation (B), Annexin V/7-AAD flow cytometric (C) and IncuCyte live cell imaging (D) data of single agent and combination ruxolitinib and venetoclax treatment of the indicated doses at 48 hours compared to a DMSO control. Two independent experiments were performed with triplicate measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

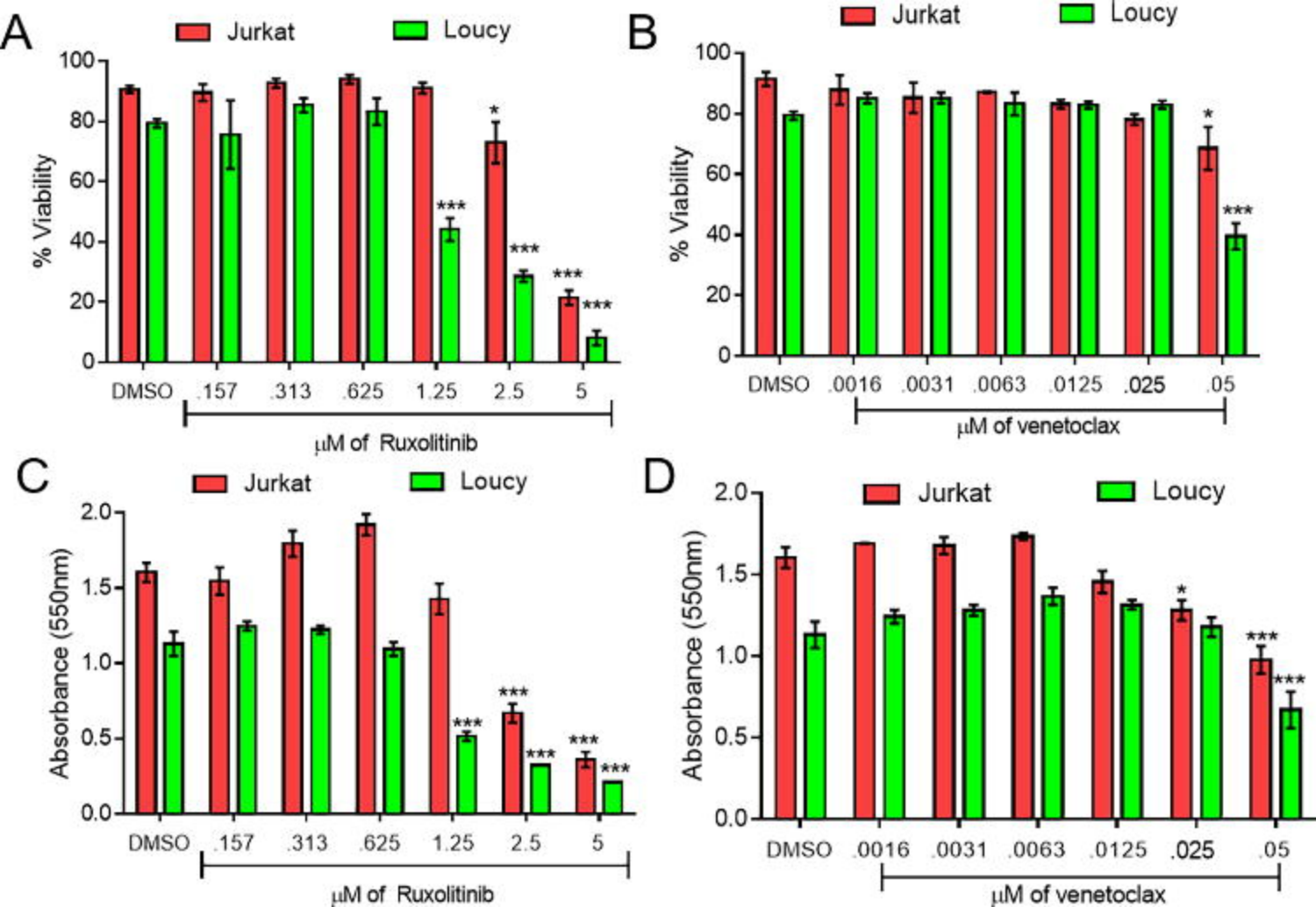
Figure 4. Ruxolitinib and venetoclax single agent and combination treatment is not efficacious against T-ALL in vivo. (A) Schematic of experimental design. (B-C) Clinical score (B) and percent survival (C) of single agent ruxolitinib, venetoclax or combination treatment given by oral gavage once daily for 14 days starting at seven days post Jurkat transplant. N=5 mice/group.

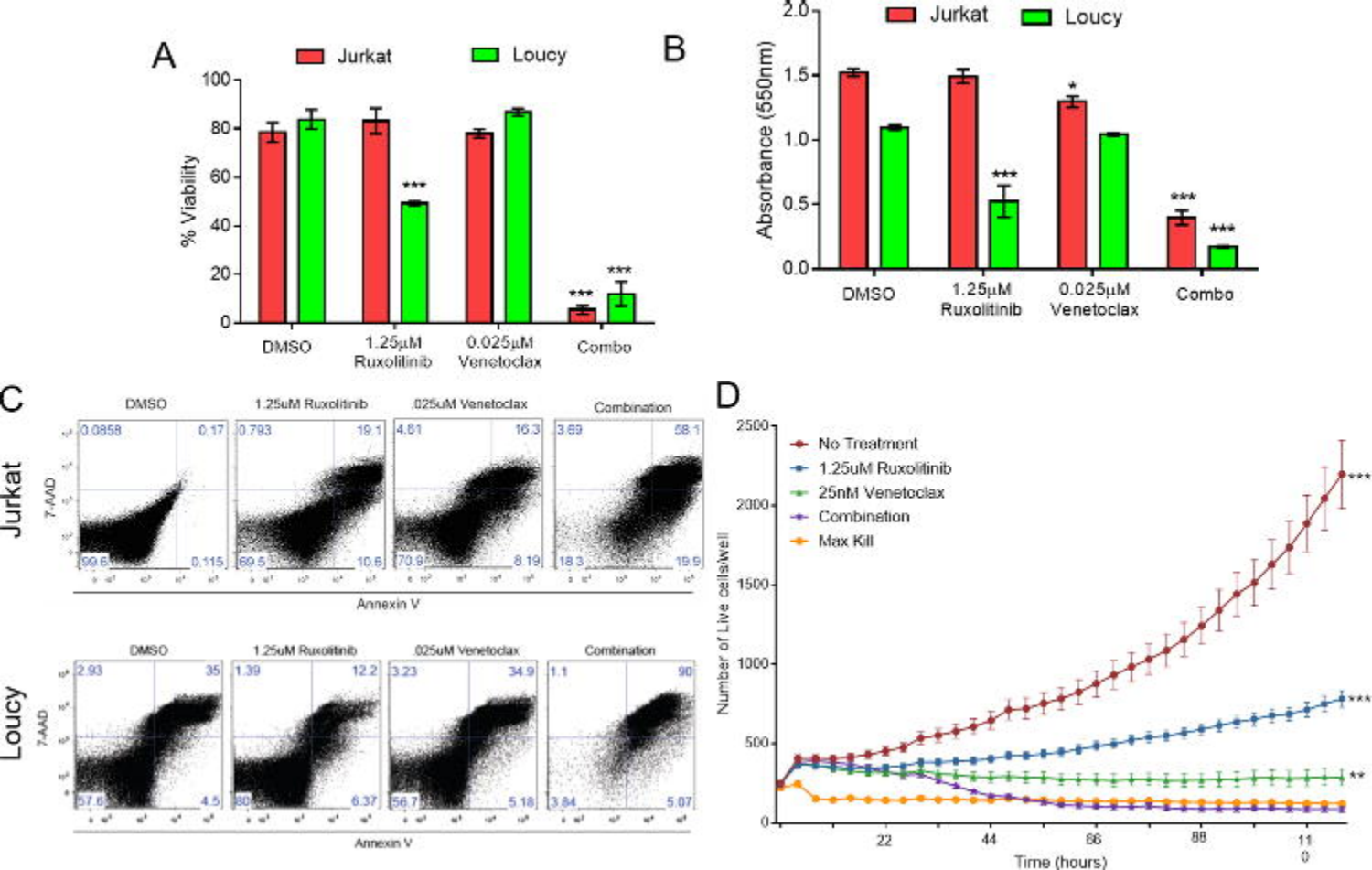
Figure 5. T-ALL evades ruxolitinib and venetoclax treatment in the CNS. (A-B) Flow cytometric analysis of human $CD45^+/GFP^+$ Jurkat cells in the bone marrow, brain, liver and spinal cord of NSG mice between 10 and 60 days post-transplant. (C) Immunohistochemistry of spleen and brain sections stained with DAPI (blue) and an anti-GFP/AF-555 secondary antibody (red). (D) LC-MS-MS analysis of ruxolitinib and venetoclax concentration in the serum, spinal

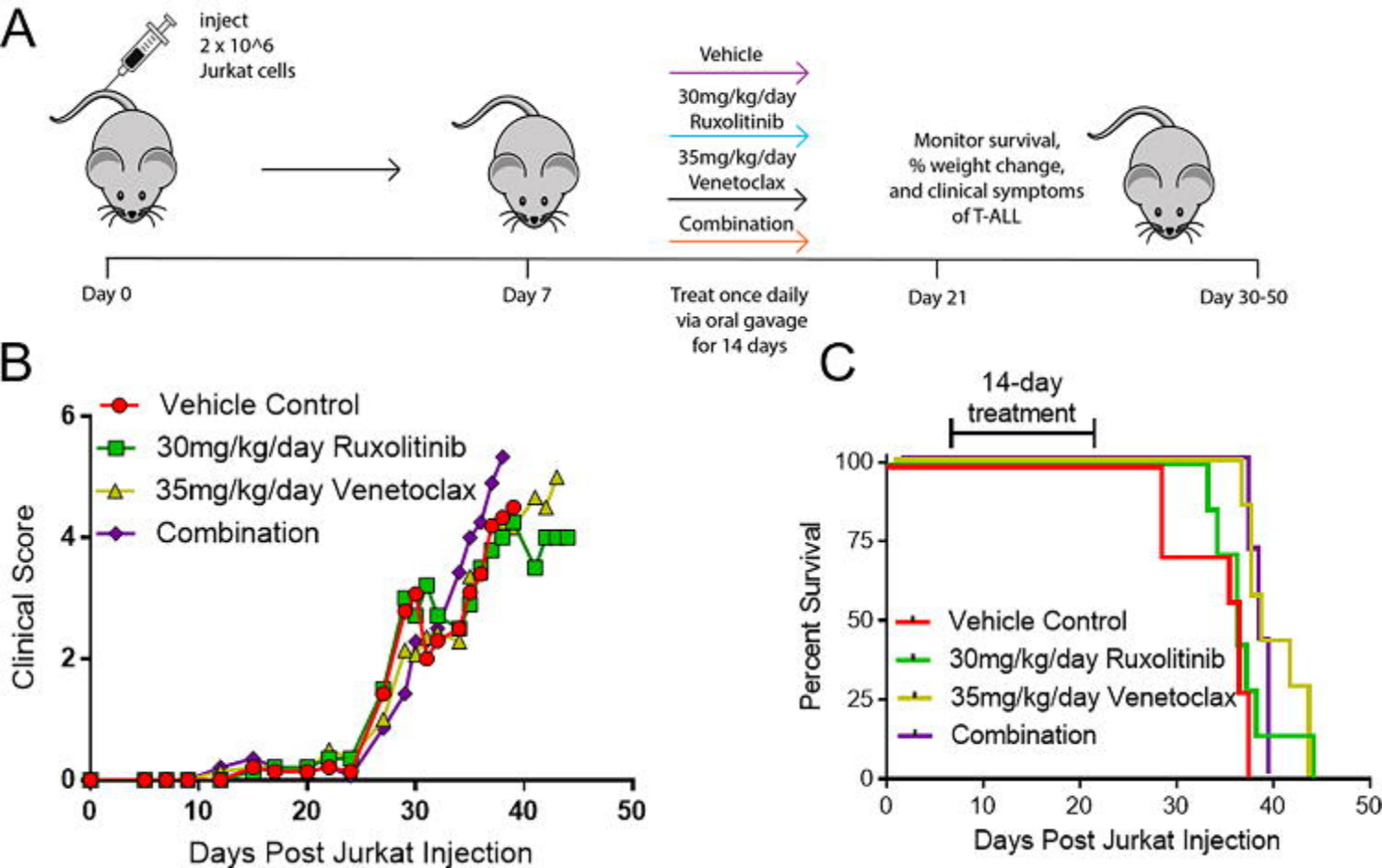
cord and brain of NSG mice after an oral gavage of 50mg/kg of ruxolitinib or 100mg/kg of venetoclax. N= 3 mice at each time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

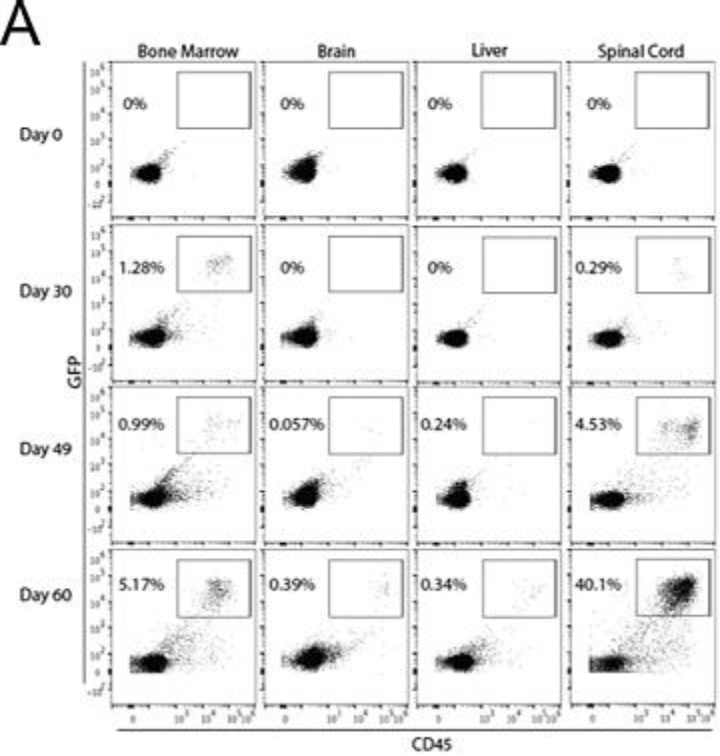
Figure 6. Blocking CXCR4 prevents CNS infiltration and facilitates combination therapy efficacy. (A) Histogram of CXCR4 expression on Jurkat cells and $CD3^+$ healthy adult T cells compared to an isotype control. Bar graph represents two independent experiments. (B) Histogram of successful CXCR4 expression reduction in Jurkat cells (blue line) compared to a no guide control (red line). (C-E) Clinical score (C), CNS clinical score according to Supplemental Table 1 (D) and overall survival (E) for control Jurkat cells (blue) and CXCR4-KO Jurkat cells (red). (F-G) Clinical score (F) and overall survival (G) are shown for mice injected with wild-type Jurkat cells and treated with either ruxolitinib/venetoclax (red line) or ruxolitinib/venetoclax and the CXCR4 inhibitor plerixafor (blue line). N=3-10 mice/group * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

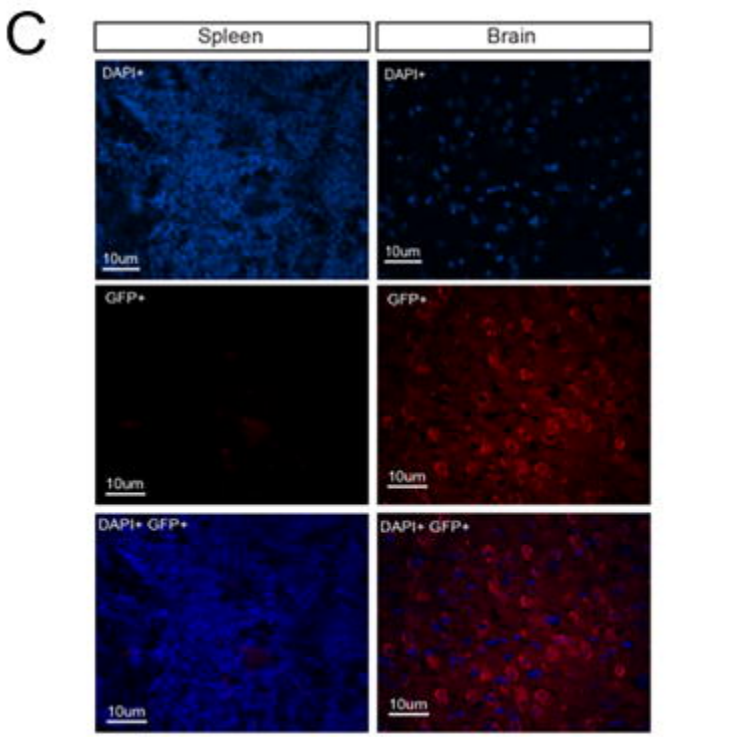

A
Healthy
Jurkat
Loucy
Isotype
pSTAT1
pSTAT1
Fold MFI
**
Jurkat
Loucy
Healthy
D
STAT1
New Diagnosis
Relapse
Normalized FPKM
New Diagnosis
Relapse
Event
B
Healthy
Jurkat
Loucy
Isotype
pSTAT3
pSTAT3
Fold MFI
**
Jurkat
Loucy
Healthy
E
STAT3
New Diagnosis
Relapse
Normalized FPKM
New Diagnosis
Relapse
Event
C
Healthy
Jurkat
Loucy
Isotype
Bcl2
Bcl2
Fold MFI
*
Jurkat
Loucy
Healthy
F
BCL2
New Diagnosis
Relapse
Normalized FPKM
*
New Diagnosis
Relapse
Event

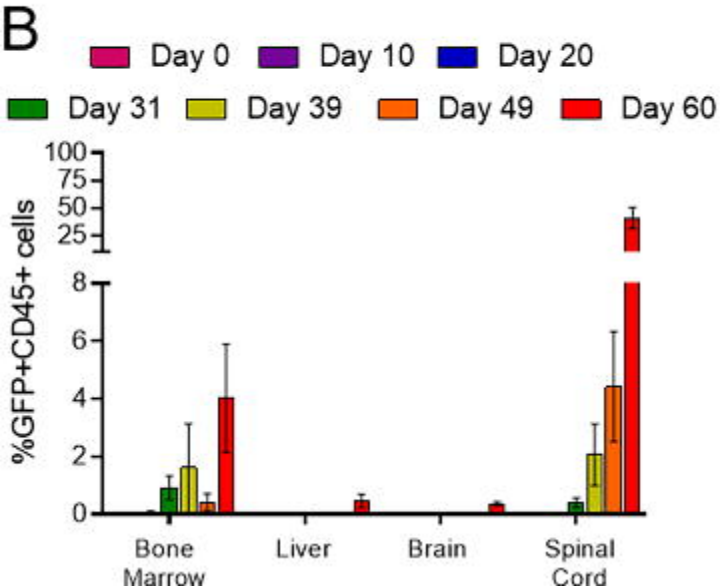

A
Jurkat
Loucy
% Viability
100
80
60
40
20
0
*
***
***
***
***
DMSO
.157
.313
.625
1.25
2.5
5
μM of Ruxolitinib
B
Jurkat
Loucy
% Viability
100
80
60
40
20
0
*
***
DMSO
.0016
.0031
.0063
.0125
.025
.05
μM of venetoclax
C
Jurkat
Loucy
Absorbance (550nm)
2.0
1.5
1.0
0.5
0.0
***
***
***
***
***
***
DMSO
.157
.313
.625
1.25
2.5
5
μM of Ruxolitinib
D
Jurkat
Loucy
Absorbance (550nm)
2.0
1.5
1.0
0.5
0.0
*
***
***
DMSO
.0016
.0031
.0063
.0125
.025
.05
μM of venetoclax

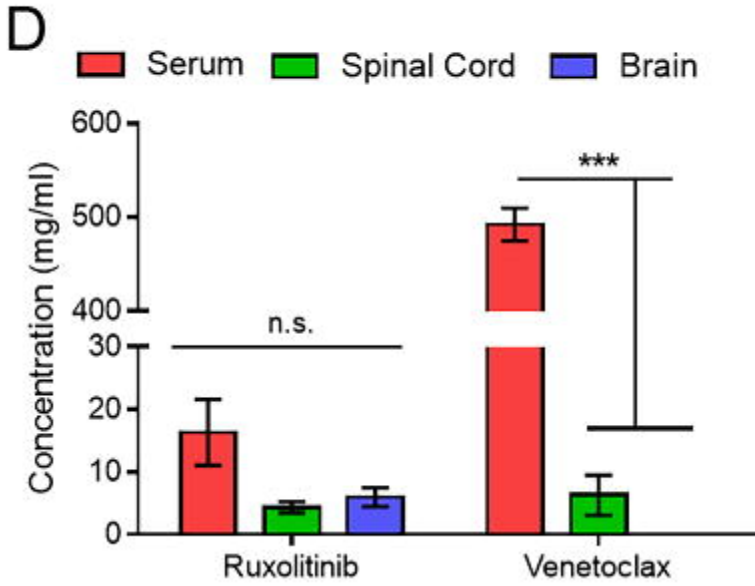

A
Jurkat
Loucy
% Viability
100
80
60
40
20
0
***
***
***
DMSO
1.25μM Ruxolitinib
0.025μM Venetoclax
Combo
B
Jurkat
Loucy
Absorbance (550nm)
2.0
1.5
1.0
0.5
0.0
*
***
***
***
DMSO
1.25μM Ruxolitinib
0.025μM Venetoclax
Combo
C
DMSO
1.25uM Ruxolitinib
.025uM Venetoclax
Combination
Jurkat
7-AAD
0.0858
0.17
99.6
0.115
0.793
19.1
69.5
10.6
4.61
16.3
70.9
8.19
3.69
58.1
18.3
19.9
Annexin V
DMSO
1.25uM Ruxolitinib
.025uM Venetoclax
Combination
Loucy
7-AAD
2.93
35
57.6
4.5
1.39
12.2
80
6.37
3.23
34.9
56.7
5.18
1.1
90
3.84
5.07
Annexin V
D
No Treatment
1.25uM Ruxolitinib
25nM Venetoclax
Combination
Max Kill
Number of Live cells/well
2500
2000
1500
1000
500
0
***
***
**
22
44
66
88
110
Time (hours)

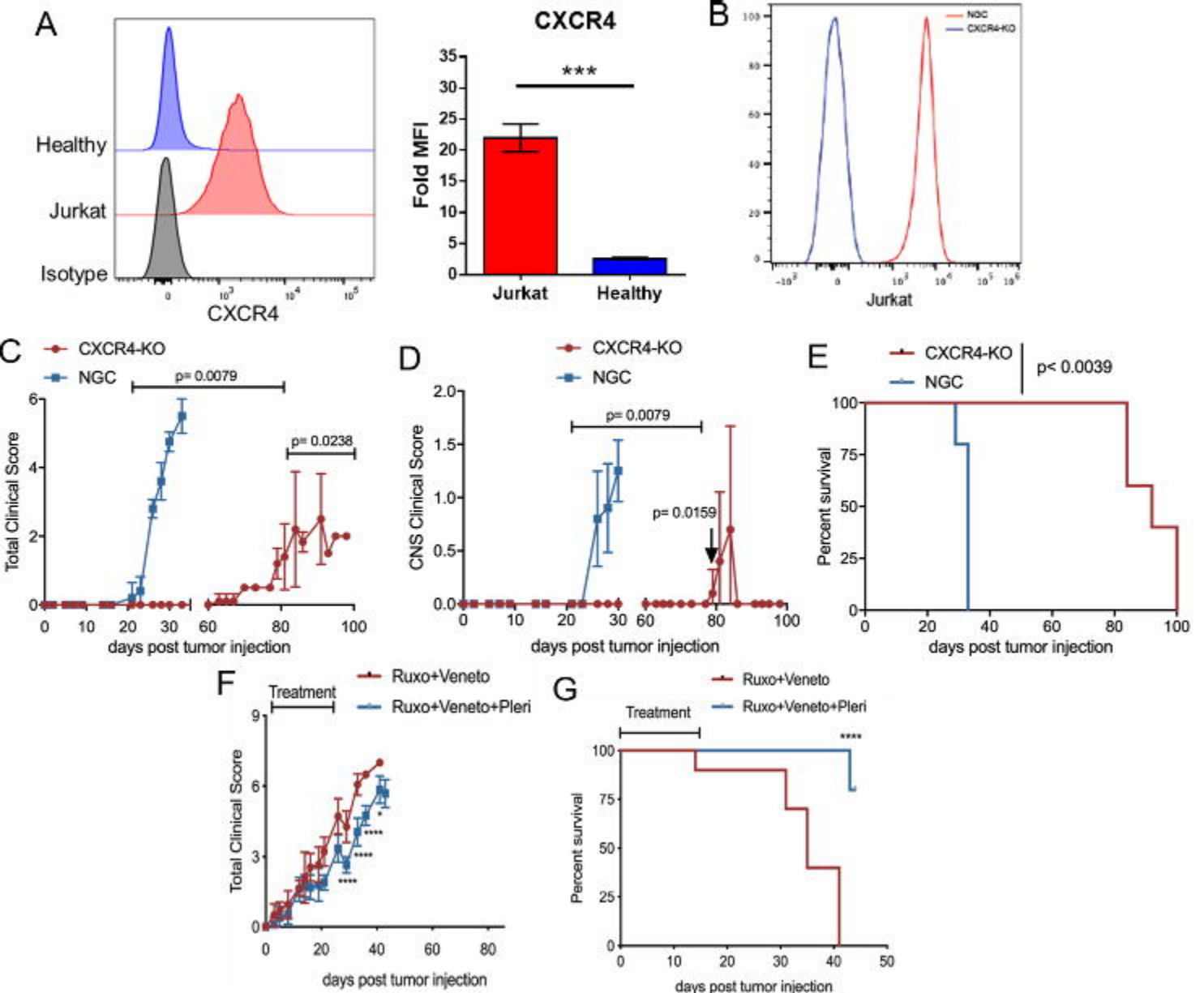

A
inject
2 x 10^6
Jurkat cells
Vehicle
30mg/kg/day
Ruxolitinib
35mg/kg/day
Venetoclax
Combination
Monitor survival,
% weight change,
and clinical symptoms
of T-ALL
Day 0
Day 7
Treat once daily
via oral gavage
for 14 days
Day 21
Day 30-50
B
Vehicle Control
30mg/kg/day Ruxolitinib
35mg/kg/day Venetoclax
Combination
Clinical Score
Days Post Jurkat Injection
C
14-day
treatment
Percent Survival
Vehicle Control
30mg/kg/day Ruxolitinib
35mg/kg/day Venetoclax
Combination
Days Post Jurkat Injection


A
Bone Marrow
Brain
Liver
Spinal Cord
Day 0
Day 30
Day 49
Day 60
GFP
CD45
0%
0%
0%
0%
1.28%
0%
0%
0.29%
0.99%
0.057%
0.24%
4.53%
5.17%
0.39%
0.34%
40.1%


C
Spleen
Brain
DAPI+
GFP+
DAPI+ GFP+
10um


B
Day 0
Day 10
Day 20
Day 31
Day 39
Day 49
Day 60
%GFP+CD45+ cells
100
75
50
25
8
6
4
2
0
Bone Marrow
Liver
Brain
Spinal Cord


D
Serum
Spinal Cord
Brain
Concentration (mg/ml)
600
500
400
30
20
10
0
n.s.
***
Ruxolitinib
Venetoclax


A
Healthy
Jurkat
Isotype
CXCR4
CXCR4
***
Fold MFI
Jurkat
Healthy
B
NGC
CXCR4-KO
Jurkat
C
CXCR4-KO
NGC
p= 0.0079
p= 0.0238
Total Clinical Score
days post tumor injection
D
CXCR4-KO
NGC
p= 0.0079
p= 0.0159
CNS Clinical Score
days post tumor injection
E
CXCR4-KO
NGC
p< 0.0039
Percent survival
days post tumor injection
F
Treatment
Ruxo+Veneto
Ruxo+Veneto+Pleri
Total Clinical Score
days post tumor injection
G
Treatment
Ruxo+Veneto
Ruxo+Veneto+Pleri
****
Percent survival
days post tumor injection