Combinatorial fedratinib and venetoclax treatment is effective on human B cell acute lymphoblastic leukemia with high Flt3 expression

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Abstract

Treatment of relapsed/refractory B cell acute lymphoblastic leukemia (B-ALL) remains a challenge, particularly in patients who do not respond to traditional chemotherapy or immunotherapy. The objective of this study was to assess the efficacy of fedratinib, a semi selective JAK2 inhibitor and venetoclax, a selective BCL-2 inhibitor, on human B-ALL using both single-agent and combinatorial treatments. The combination treatment of fedratinib and venetoclax improved killing of the human B-ALL cell lines RS4;11 and SUPB-15 in vitro over single-agent treatments. This combinatorial effect was not detected in the human B-ALL cell line NALM-6, which was less responsive to fedratinib due to the absence of Flt3 expression. The combination treatment induces a unique gene expression profile relative to single-agent treatment and with an enrichment in apoptotic pathways. Finally, the combination treatment was superior to single agent treatment in an in vivo xenograft model of human B-ALL with a two-week treatment regimen significantly improving overall survival. Overall, our data demonstrates the

30 efficacy of a combinatorial treatment strategy of fedratinib and venetoclax against
31 human B-ALL expressing high levels of Flt3.
32
33 **Key points**
34 1. Combination fedratinib and venetoclax reduces the survival and proliferation of
35 $FLT3^+$ B-ALL in vitro.
36 2. Gene set enrichment analysis of RNA from B-ALL treated with fedratinib and
37 venetoclax identified dysregulation of pathways associated with apoptosis, DNA
38 repair and proliferation.
39 3. Combination fedratinib and venetoclax reduces the number of peripheral blood
40 B-ALL blasts in vivo, improving overall survival while also increasing CD19
41 expression.
42
43 **Introduction**
44 Pediatric B cell acute lymphoblastic leukemia (B-ALL) patients who relapse after initial
45 treatment suffer from a poor prognosis (1,2). While many relapsed/refractory pediatric
46 patients are salvaged with CD19-directed immunotherapies like blinatumomab or
47 tisagenlecleucel, the 12 month event free survival is only 50%, with CD19 loss
48 representing a major barrier to the long-term success of these immunotherapies (3).
49 Many of these immunotherapies are also associated with a high cost, making them
50 unattainable to some patients. It is thus essential that we investigate novel
51 combinatorial treatments with targeted pharmaceuticals to improve patient outcomes
52 and quality of life.
53
54 The dysregulation and/or activating mutations in the protein Janus kinase 2 (JAK2) lead
55 to downstream phosphorylation and activation of signal transducer and activator of
56 transcription (STAT) proteins that are predominant in many cancers (4,5). In a recent
57 Children's Oncology Group (COG) study (6) genomic alterations involving activation of
58 the JAK/STAT pathway were found in 22% of high-risk B cell ALL patient samples,
59 including Philadelphia (Ph)-like B-ALL.
60

61 The BCL-2 family of proteins are central regulators of apoptosis. BCL-2 inhibits
62 apoptosis by the preservation of mitochondrial membrane integrity, specifically
63 preventing the pro-apoptotic proteins BAX and BAK from release into the mitochondria
64 membrane and the subsequent release of cytochrome C (7). Furthermore, some high-
65 risk subsets of B-ALL, such as KMT2A-rearranged B-ALL or B-ALL with hypodiploidy,
66 express high levels of BCL-2 to prevent apoptosis (8). We and others have previously
67 shown that dual targeting of the JAK/STAT pathway and BCL-2 can treat acute
68 myelogenous leukemia (9) and T cell ALL (10), however the impact of targeting both
69 these pathways in B-ALL is unknown.
70
71 The overall goal of this study was to investigate the direct anti-leukemic activity of the
72 JAK2 inhibitor fedratinib and the BCL-2 inhibitor venetoclax either as a single-agent
73 treatment or combinatorial treatment. By inhibiting both of these pathways at the same
74 time, we aim to enhance their anti-leukemic effects on B-ALL.
75
76 **Materials and Methods:**
77
78 B-ALL cell lines
79 NALM-6 (B-ALL), RS4;11 (KMT2A-rearranged B-ALL) and SUP-B15 (Ph-like B-ALL)
80 were purchased from ATCC. NALM-6 and RS4;11 were cultured in RPMI 1640
81 (Corning) supplemented with 10% fetal bovine serum (FBS) (GeminiBio), 2 mmol/L L-
82 glutamine (Gibco), and 100 mg/mL streptomycin/100 U/mL penicillin (Lonza). SUP-B15
83 cells were cultured in McCoy 5A (modified) media (Gibco) supplemented with 20% FBS,
84 100 mg/mL streptomycin and 100 U/mL penicillin. RS4;11 cells were transduced to
85 express the luminescent reporter luciferase using pGL4.51[luc2/CMV/Neo] vector
86 (Promega). Cell authentication was performed using short tandem repeat analysis
87 (Idexx BioAnalytics, Westbrook, ME) and per ATCC guidelines using morphology,
88 growth curves, and *Mycoplasma* testing within 6 months of use (InVivoGen). Cell lines
89 were maintained in culture at 37°C in 5% CO_2.
90
91 Fedratinib and Venetoclax

92 Venetoclax (1 mL*10 mM in DMSO) and powdered compound were purchased from
93 TargetMol. Fedratinib (1 mL*10 mM in DMSO) was purchased from Selleckchem and
94 powdered was purchased from MedChemExpress. All concentrations for in vitro
95 studies were made at 2x or 4x for combination studies and diluted in RPMI-10 for
96 plating assays. Cells were treated with a range of doses ranging from 1nM to 10uM.
97
98 Live/dead discrimination assays
99 Cells were collected after a 48 hour incubation with either vehicle control, fedratinib,
100 venetoclax, or combination and live/dead discrimination was assessed through flow
101 cytometry. Samples were labeled with Ghost Dye Red 780 (Tonbo Biosciences) and
102 analyzed via an Attune NxT Flow Cytometer (ThermoFisher) and analysis on FlowJo
103 V10.
104
105 Proliferation assays
106 Cells were collected after a 72 hour incubation with either vehicle control, fedratinib,
107 venetoclax, or combination, and then proliferation was assessed through a colorimetric
108 MTS assay for determining cell viability. The CellTiter 96 AQueous One Solution Cell
109 Proliferation Assay (Promega) was used and 96 well plates were read using a
110 CLARIOStar microplate reader (BMG Labtech).
111
112 RNA seq
113 RS4;11 cells were treated for 24 hours in triplicate (untreated, fedratinib alone,
114 venetoclax alone, or combination). All RNA isolation and sequencing was performed by
115 the Gene Expression Center at the University of Wisconsin-Madison. RNA was isolated
116 with a RNeasy kit (Qiagen) prior to a quality control check using an Agilent Bioanalyzer
117 and NanoDrop One spectrophotometer. mRNA libraries were prepared using the
118 TruSeq stranded mRNA kit and sequenced on an Illumina NovaSeq 6000 platform to 30
119 million reads per sample. Sample reads were processed by the University of Wisconsin
120 Biotechnology Center.
121
122 In vivo studies

123 NOD/SCID/*Il2rg*^{tmwjl}/Szj (NSG) breeder mice were purchased from Jackson Labs (Bar
124 Harbor, ME) and bred internally at the Biomedical Research Model Services (BRMS)
125 Breeding Core at the University of Wisconsin-Madison in accordance with the Guide for
126 the Care and Use of Laboratory Mice and experiments were performed under an IACUC
127 approved animal protocol (M005915). Male and female NSG mice were used at 8-16
128 weeks of age and injected via tail vein with 2 x10^6 RS4;11 cells on Day 0. Peripheral
129 blood was obtained via retro-orbital bleeds conducted on Days 10 (engraftment), Day
130 21 (pre-treatment), and Day 35 (post-treatment). Mice were treated with fourteen days
131 of once daily treatment with vehicle, fedratinib, venetoclax, or combination starting at
132 Day 21 (5 days on, 2 days off). Venetoclax was administered via intraperitoneal (IP)
133 injections at a dose of 6.25 mg/kg. Fedratinib was administered via oral gavage at a
134 dose of 100 mg/kg. Mice were monitored for survival and percent weight change and
135 euthanized when a humane endpoint was reached. Peripheral blood was treated with
136 ACK lysis buffer (Lonza) and then stained in flow buffer (PBS, 10% FBS) with CD19
137 (HIB19), CD20 (2H7), CD22 (HIB22), PD-L1 (MIH2), FAS (DX2), HLA-DR (L243), BCL2
138 (100) and BAX (2D2), all purchased from Biolegend, prior to quantification on an Attune
139 NxT Flow Cytometer with analysis on FlowJo V10.
140
141 <u>RNA-Seq analysis</u>
142 Bioinformatic analysis of transcriptomic data adhere to recommended ENCODE
143 guidelines and best practices for RNA-Seq. Alignment of adapter-trimmed (Skewer
144 v0.1.123; (11)) 2x150 (paired-end; PE) bp strand-specific Illumina reads to the *Homo*
145 *sapiens*GRCh38 genome (assembly accession NCBI:GCA_000001405.25) was
146 achieved with the Spliced Transcripts Alignment to a Reference (STAR v2.5.3a)
147 software (12), a splice-junction aware aligner, using annotation provided by Ensembl
148 release 104. Expression estimation was performed with RSEM v1.3.0 (RNASeq by
149 Expectation Maximization; (13)). To test for differential gene expression among
150 individual group contrasts, expected read counts obtained from RSEM were used as
151 input into edgeR (v3.16.5; (14)). Inter-sample normalization was achieved with the
152 trimmed mean of M-values (TMM; (15)) method. Statistical significance of the negative-
153 binomial regression test was adjusted with a Benjamini-Hochberg FDR correction at the

154 5% level (16). Prior to statistical analysis with edgeR, independent filtering was applied
155 and required genes to have a count-per-million (CPM) above *k* in *n* samples, where *k* is
156 determined by minimum read count (10 reads) and by the sample library sizes
157 where *n* is determined by the number of biological replicates in each group. The validity
158 of the Benjamini-Hochberg FDR multiple testing procedure was evaluated by inspection
159 of the uncorrected p-value distribution.
160

161 Statistical analysis

162 Statistics were performed using GraphPad Prism version 9.0 for the Macintosh OS
163 (GraphPad Software). Data were expressed as mean ± SEM. For analysis of three or
164 more groups, a non-parametric one-way ANOVA test was performed with the Bonferroni
165 or Sidak's multiple comparisons post-test. Analysis of differences between two normally
166 distributed test groups was performed using a two-sided Mann Whitney test. A p-value
167 less than 0.05 was considered statistically significant.

168

169 **Results:**

170

171 *Combinatorial treatment of fedratinib and venetoclax effectively kills human B-ALL in*
172 *vitro*

173

174 To assess the efficacy of JAK inhibitors to kill human B-ALL cells, we tested ruxolitinib
175 (JAK1/2 inhibitor), lestaurtinib (semi-selective JAK2 inhibitor) and fedratinib (semi-
176 selective JAK2 inhibitor) on the human B-ALL cell line RS4;11. Cells were treated with a
177 range of doses ranging from 1nM to 10uM for 48-72 hours prior to analysis for cell
178 survival (Figure 1A-B, Suppl Figure 1). Survival and proliferation of RS4;11 was
179 assessed by flow cytometry at 48hrs or through a colorimetric MTS assay (Figure 1A-
180 B). Based on the dose-curves, the calculated IC50 for lestaurtinib was 60nM, fedratinib
181 was 600nM but the IC50 for ruxolitinib was not reached.
182

183 We next wanted to explore whether or not fedratinib would be more efficacious when
184 combined with venetoclax. To test this, we treated RS4;11 with single agent fedratinib at
185 700nm, single agent venetoclax at 7.5nM and the combinatorial treatment (Figure 1D-
186 E). Combinatorial treatment was significantly more effective than either single agent
187 treatment and similar to the positive control staurosporine. We next tested if ruxolitinib
188 had a similar beneficial effect as fedratinib (Fig 1F-G). Ruxolitinib, used at a higher dose
189 than fedratinib, did not improve the killing of RS4;11 over single-agent venetoclax (Fig
190 1F-G). To ensure that the combinatorial effect of fedratinib and venetoclax was not
191 specific to RS4;11, we tested both the single agent and combinatorial treatments on the
192 human B-ALL cell lines SUPB-15 and NALM-6. The combinatorial treatment was
193 equally effective on SUPB-15 (Fig 2A-B) but surprisingly, the combinatorial treatment
194 did not improve the killing of NALM-6 cells compared to venetoclax alone (Fig 2C-D).
195 These data suggest that while the combinatorial treatment of fedratinib and venetoclax
196 can be highly effective in human B-ALL, there are a subset of B-ALLs that remain
197 resistant to this combinatorial treatment.
198
199 *Resistance to fedratinib treatment is correlated with absence of Flt3 expression*
200
201 To determine the precise activity of and contribution to cell killing by fedratinib and
202 venetoclax, we performed several additional dose-escalation studies on all three human
203 B-ALL cell lines. Keeping a constant venetoclax dose of 5 nM, both RS4;11 and SUPB-
204 15 experienced significant cell death that was further enhanced with increasing doses of
205 fedratinib. Conversely, NALM-6 was mostly refractive to venetoclax at 5 nM with
206 increasing concentrations of fedratinib only modestly improving the killing efficiency (Fig
207 3A-C, left panels). Using a constant dose of fedratinib of 525 nM, increasing doses of
208 venetoclax was able to significantly increase cell death in both RS4;11 and SUP-B15
209 while NALM-6 remains refractory as before (Fig 3 A-C, right panels).
210
211 To assess a potential mechanism for NALM-6 resistance to both venetoclax and
212 fedratinib treatment, we measured the expression of BCL-2, BRD4 and FLT3 in each
213 human B-ALL cell line. Notably, NALM-6 had no expression of FLT3, which is also a

214 target of fedratinib while RS4;11, which has the greatest sensitivity to fedratinib, had the
215 highest expression of Flt3 (Fig 3D-E). We also observed that NALM-6 was more
216 resistant to venetoclax treatment than either RS4;11 or SUPB-15 (Fig 1, 2).
217 Interestingly, NALM-6 had a significantly lower ratio of the pro-apoptotic protein BAX to
218 the anti-apoptotic protein BCL2 relative to RS4;11, which correlates with their
219 venetoclax sensitivity (Fig 3F).
220
221 *Combination therapy differentially enriches pathways related to cell death*
222
223 Next, we wanted to identify the dominant pathways altered in RS4;11 post single-agent
224 and combination treatment via bulk RNA sequencing. RS4;11 cells were plated similarly
225 as described above and exposed for 24 hours to fedratinib (525 nM), venetoclax (5 nM),
226 or combination and compared to untreated cells. Cells were harvested after 24 hours for
227 RNAseq. Whole transcriptome analysis revealed that each treatment regimen induced a
228 unique expression profile clustered image map (Fig 4A-B). Comparing each treatment
229 regimen to untreated cells, fedratinib had the fewest number of significantly different
230 genes while the combinatorial treatment had the most (Fig 4C-E). Interestingly in all
231 cases, most of the differentially expressed genes were down-regulated and not up-
232 regulated as a result of treatment (Fig 4C-E). We also completed a gene set enrichment
233 analysis (GSEA) that identified pathways associated with apoptosis, DNA repair and
234 proliferation predominantly altered in the combinatorial treatment versus all three other
235 conditions (Fig 4F). A heatmap of the differentially enriched genes primarily impacting
236 the enrichment into the apoptotic pathway is also shown (Fig 4G).
237
238 *Combination therapy reduces leukemic burden and improves overall survival in RS4;11*
239 *derived xenograft model of B-ALL*
240
241 Based on the efficacy of fedratinib and venetoclax observed *in vitro*, a xenograft model
242 of B-ALL was established utilizing RS4;11 cells and NSG mice to test these drugs as a
243 combination therapy *in vivo*. NSG mice were injected with luciferase^{+} RS4;11 cells on
244 Day 0 and then randomized to one of the following groups: untreated, single agent

245 venetoclax, single agent fedratinib, or combination. Peripheral blood was obtained via
246 retroorbital bleeds on days 10 (engraftment), 21 (pretreatment), and 35 (post treatment)
247 to assess B-ALL disease burden (Fig 5A). Treatment started on day 21 and lasted for
248 14 days (5 days on, 2 days off) with 6.25 mg/kg venetoclax IP and/or 100 mg/kg
249 fedratinib via gavage. Combination treatment prolonged the survival of the mice and
250 decreased the RS4;11 burden the most compared to the other treatment groups (Fig
251 5B). Peripheral blood counts and IVIS imaging throughout the experiment revealed that
252 combination treatment was highly effective at decreasing the RS4;11 burden in vivo (Fig
253 5C-F). Venetoclax alone also significantly improved overall survival when compared to
254 fedratinib alone and untreated mice (Figure 5B-F). Flow cytometric analysis of RS4;11
255 taken from the peripheral blood also showed a significant increase in MFI of FAS and
256 CD19 in the combination group. Overall, these data support the role of fedratinib and
257 venetoclax as a combination therapy against $FLT3^+$ B-ALL.
258

259 **Discussion:**

260

261 Treatment of relapsed/refractory B cell acute lymphoblastic leukemia (B-ALL) remains a
262 challenge, particularly in patients who do not respond to traditional chemotherapy or
263 immunotherapy. Targeting BCL-2 in B-ALL has been shown previously to be efficacious
264 using venetoclax. One group showed a single dose of 12.5 mg/kg venetoclax in a
265 RS4;11-derived xenograft model led to a maximal tumor growth inhibition of 47% and
266 tumor growth delay of 26%. However, maximal effects were seen when combining
267 venetoclax with chemotherapy (17). Other studies demonstrating efficacy of venetoclax
268 in B-ALL include combining with MCL-1 inhibitors (18, 19), monoclonal antibodies such
269 as daratumumab in relapsed/refractory B-ALL (20), AKT inhibitors (21, 22), MDM2
270 inhibitors (23), and also with BCR-ABL positive B-ALL (24). No other studies have
271 previously looked at the combination of JAK2 inhibitors with venetoclax in B-ALL. The
272 dysregulation of JAK/STAT related genes has been previously shown to be found in
273 high risk patient samples of B-ALL (6). One group identified rearrangements of JAK2 in

274 Ph like B-ALL involving the activation of mutations in IL7R and FLT3, and deletion of
275 SH2B3, which encodes the JAK2 negative regulator LNK (25).
276
277 The goal of this study was to suppress leukemia proliferation related to JAK/STAT
278 dysregulation while at the same time inhibiting BCL-2 to prevent escape from apoptosis
279 in models of human B-ALL. To do this, we tested fedratinib, a semi selective JAK2
280 inhibitor, and venetoclax, a potent BCL2 inhibitor, to assess the efficacy of this
281 combination in 3 models of human B-ALL in vitro and with the RS4;11 cell line in vivo.
282 Fedratinib has been FDA approved for patients with myeloproliferative disorders,
283 primarily myelofybrosis (26, 27) and has been used to salvage patients that have failed
284 post ruxolitinib therapy (28). Furthermore, fedratinib has been used to decrease tumor
285 burden in Hodgkin lymphoma and mediastinal large B-cell lymphoma in vitro and in vivo
286 (29). However, fedratinib has not previously been used for B-ALL. Analysis of BRD4
287 and FLT3 was included for all 3 cell lines in our study as fedratinib has been shown to
288 target both BRD4 (30, 31) and FLT3 (30) in addition to its activity against JAK2.
289
290 Our data both in vitro and in vivo demonstrated that the combination of fedratinib and
291 venetoclax more effectively treated B-ALL than single agent treatment. We also
292 examined the use of other JAK inhibitors, including ruxolitinib, which we had previously
293 shown synergized with venetoclax to treat T cell ALL (10). Similar to observations by
294 others (18), we found that ruxolitinib alone was ineffective in doses up to 10 uM. That
295 group did demonstrate that there may be some efficacy when ruxolitinib was combined
296 with venetoclax in Ph+ B-ALL models of disease in vitro. Combination fedratinib and
297 venetoclax did demonstrate efficacy in another high-risk Ph+ B-ALL cell line SUP-B15,
298 perhaps suggesting that the combination of targeting the JAK/STAT and BCL-2
299 pathways simultaneously is particularly effective in Ph-like and FLT3+ high risk subsets
300 compared to more standard B-ALL models of disease such as NALM-6. Furthermore,
301 lestaurtinib, another semi selective JAK inhibitor was tested in preliminary studies.
302 Lestaurtinib, similar to fedratinib, also inhibits FLT3 which is commonly overexpressed
303 in KMT2A-rearranged B-ALL (32) and was found to be highly expressed in RS4;11. The
304 association of FLT3 and BCL2 in B-ALL is unclear but our RNA GSEA suggest the

305 potential roles of proliferation, apoptosis and DNA repair pathways with combination
306 fedratinib and venetoclax treatment.
307
308 Finally, we demonstrated efficacy of combination fedratinib and venetoclax therapy in a
309 B-ALL xenograft model. In fact, with only a 2-week treatment we were able to identify
310 reduced leukemic burden and an increase in overall survival benefit with combination
311 therapy. Our group attempted to reduce potential toxicity in our in vivo model by
312 reducing DMSO concentration in our venetoclax drug preparation for in vivo use and
313 gavaging with sucrose-soaked gavage tips to reduce mice stress as much as possible
314 using techniques described by others (33). Our group also identified an increase in
315 CD19 expression in peripheral blood samples obtained from mice that received either
316 venetoclax or combination therapy. One group recently demonstrated pre-sensitizing B
317 cell malignancies, including B-ALL, through venetoclax exposure increases cytotoxic
318 efficacy of CD19 directed CAR-T therapy (34). These findings along with our data with
319 combination venetoclax plus fedratinib suggest these targeted therapies may serve as a
320 potential bridge therapy to sensitize B-ALL to CD19 targeted treatments such as
321 blinatumomab or chimeric antigen receptor (CAR) T cells.
322
323 The development of novel treatment strategies for relapsed/refractory B-ALL is essential
324 for the subset of patients with poor outcomes due to failure to respond to initial
325 conventional chemotherapy. The combination of fedratinib and venetoclax is an
326 effective treatment strategy to reduce the burden of disease, particularly in $FLT3^+$ B-
327 ALL, through reduction in proliferation, apoptosis and DNA repair pathways.
328 Combination fedratinib and venetoclax may also potentially serve as a bridge to CD19-
329 directed therapies such as CAR-T cell and future studies should explore if these
330 therapies can help B-ALL avoid potential CD19 antigen escape.
331
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333
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Author contributions:

SPR, NJH and CMC designed the experiments, analyzed and interpreted results and wrote the manuscript; SPR, NJH, HCB, LS, NMH, LR, DPT, and NJH conducted experiments and analyzed data; SPR, NJH, DPT and HCB analyzed data and generated figures; and SPR, NJH, and HCB discussed and interpreted results. All authors read and approved the manuscript.

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501 **Figure 1. Fedratinib, but not ruxolitinib, combines with venetoclax to improve**
502 **killing of human B-ALL**
503 (A-C) The human B-ALL cell line RS4;11 was treated with a dose curve of single agent
504 ruxolitinib (A), fedratinib (B) or venetoclax (C) and number of live cells quantified by flow
505 cytometry after 48hrs. (D-G) RS4;11 cells were treated with the indicated concentration
506 of fedratinib and venetoclax (D-E) or ruxolitinib and venetoclax (F-G) for 48hrs. Live
507 cells were quantified by flow cytometry and proliferation rate measured by colorimetric
508 MTS assay. Fed = fedratinib. Rux = ruxolitinib. Ven = venetoclax.
509

510 **Figure 2. Combinatorial treatment of venetoclax and fedratinib is efficacious in**
511 **the human B-ALL cell line SUP-B15, but not NALM-6**
512 Human B-ALL cell lines NALM-6 and SUP-B15 were treated with venetoclax in
513 combination with fedratinib. Live cells were quantified by flow cytometry as previously
514 described (A,C) and proliferation was assessed by an MTS assay as previously
515 described (B,D).
516

517 **Figure 3. Fedratinib activity is associated with Flt3 expression**
518 (A-C) Combination dose curves were performed on RS4;11 (A), SUP-B15 (B) and
519 NALM-6 (C), keeping either the venetoclax dose (left panel) or fedratinib dose (right
520 panel) constant to determine the potency of the combinatorial treatment on different
521 human B-ALL cell lines. (D-F) Expression of both Flt3 and Brd4 (D-E) and the ratio of
522 BAX to BCL2 (F) were measured in each cell line by flow cytometry. Fold MFI
523 represents the median fluorescent intensity of the marker divided by the isotype control.
524 *** $p < 0.001$.
525
526 **Figure 4. Combination treatment induces a unique gene expression profile**
527 RS4;11 cells were left untreated or treated with 5 nM ventoclax, 525 nM fedratinib or the
528 combination treatment for 24 hrs prior to RNA isolation and bulk sequencing. (A-B)
529 Transcriptome analysis of each treatment group by two-dimensional multidimensional
530 scaling (MDS) (A) and a clustered image map reveals each treatment group induces a
531 unique transcriptomic profile. (C-E) Volcano plots showing the number of positively and
532 negatively regulated genes in each treatment group relative to the untreated control. (F)
533 Gene set enrichment analysis of the combination treatment against all other treatments
534 was performed with three gene sets shown. (G) Heatmap showing the genes
535 contributing to the enrichment score of the apoptosis gene set.
536
537 **Figure 5. Fedratinib and venetoclax combination treatment is superior to single-**
538 **agent treatment in vivo**
539 (A) RS4;11 was transplanted into non-irradiated NSG mice via tail vein injection at 2E6
540 cells per mouse and allowed to engraft for 21 days prior to starting treatment.
541 Venetoclax was given IP at a dose of 6.25 mg/kg while fedratinib was given gavage at a
542 dose of 100 mg/kg. Retro-orbital blood draws were taken at day 10, 21 and 35 to
543 assess cancer burden. (B) Kaplan-meier survival curve of each treatment. (C-D)
544 Representative dot plot of RS4;11 taken from the blood at day 35 (C) and quantification
545 across all times (D). (E-F) Representative IVIS images (E) and total flux quantification of
546 all mice at the indicated time points (F). (G) A select number of B-ALL cancer markers
547 expression profiles were assessed at day 35 across all treatment conditions. Fold MFI

548 represents the median fluorescent intensity of the marker divided by the isotype control.
549 Data is representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p <$
550 0.001.
551
552 **Supplementary Figure 1. Single agent lestaurtinib and in combination with**
553 **venetoclax show efficacy against RS4;11**
554 The human B-ALL cell line RS4;11 was treated with single agent lestaurtinib for 48hrs
555 (A) or in combination with venetoclax (B) and live cells quantified by flow cytometry.
556
557 **Supplementary Figure 2. FISH of bone marrow and spinal cord samples from**
558 **untreated Day 51 RS4;11 transplanted NSG mice show very limited CNS infiltrate**
559 **during late stage leukemic disease**
560 Bone marrow and spinal cord tissue were harvested on untreated Day 51 transplanted
561 RS4;11 NSG mice. Human and mouse CEP probes via FISH were used to quantify
562 human RS4;11 cells versus mouse cells to determine disease burden in bone marrow
563 and spinal cord tissues and to confirm level of CNS infiltrate for subsequent treatment
564 with small molecule inhibitors.
565
566

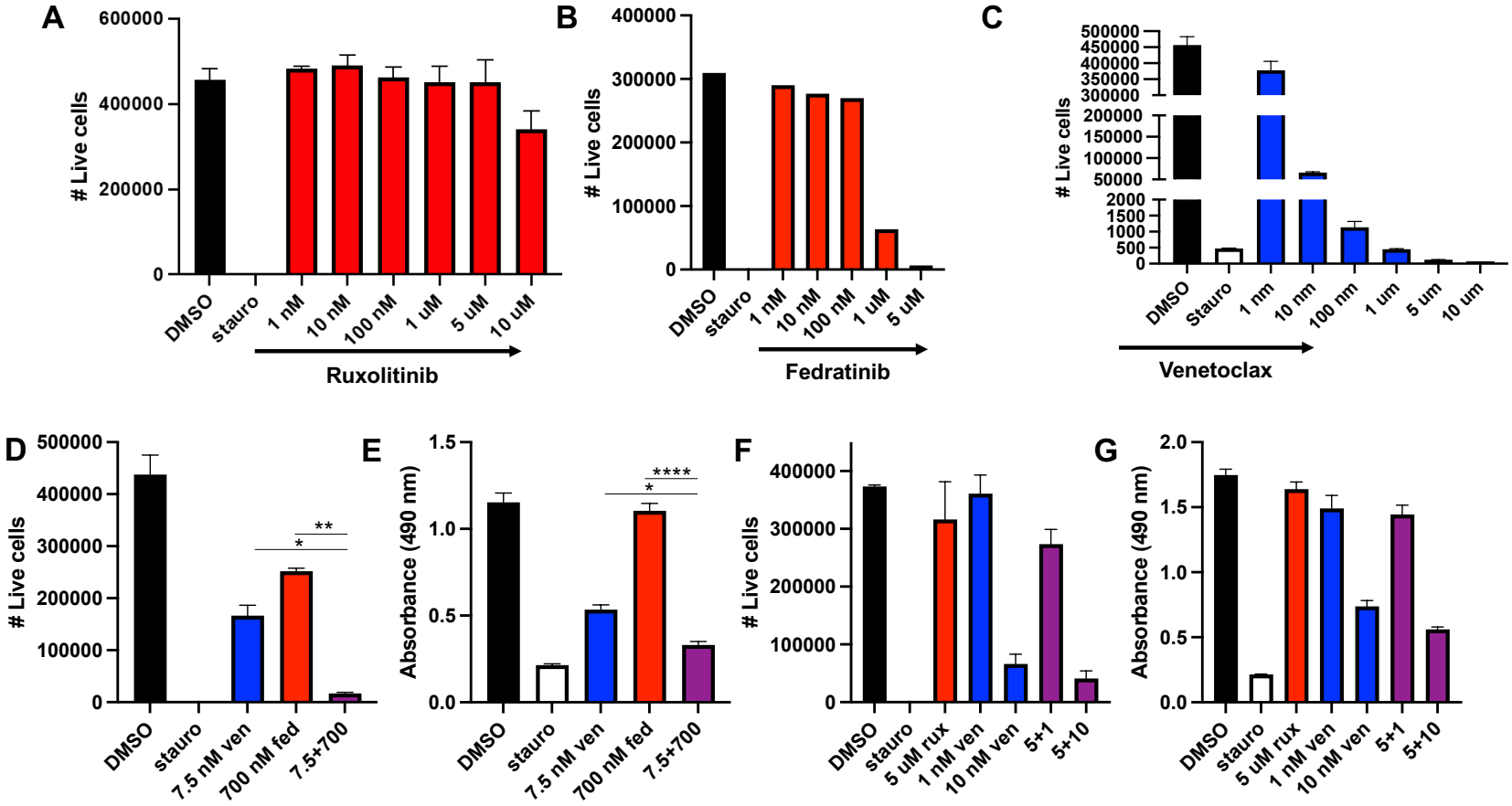
A
600000
400000
200000
0
Live cells
DMSO
stauro
1 nM
10 nM
100 nM
1 uM
5 uM
10 uM
Ruxolitinib
B
400000
300000
200000
100000
0
Live cells
DMSO
stauro
1 nM
10 nM
100 nM
1 uM
5 uM
Fedratinib
C
500000
450000
400000
350000
300000
200000
150000
100000
50000
2000
1500
1000
500
0
Live cells
DMSO
Stauro
1 nm
10 nm
100 nm
1 um
5 um
10 um
Venetoclax
D
500000
400000
300000
200000
100000
0
Live cells
**
*
DMSO
stauro
7.5 nM ven
700 nM fed
7.5+700
E
1.5
1.0
0.5
0.0
Absorbance (490 nm)
****
*
DMSO
stauro
7.5 nM ven
700 nM fed
7.5+700
F
400000
300000
200000
100000
0
Live cells
DMSO
stauro
5 uM rux
1 nM ven
10 nM ven
5+1
5+10
G
2.0
1.5
1.0
0.5
0.0
Absorbance (490 nm)
DMSO
stauro
5 uM rux
1 nM ven
10 nM ven
5+1
5+10

Figure 1

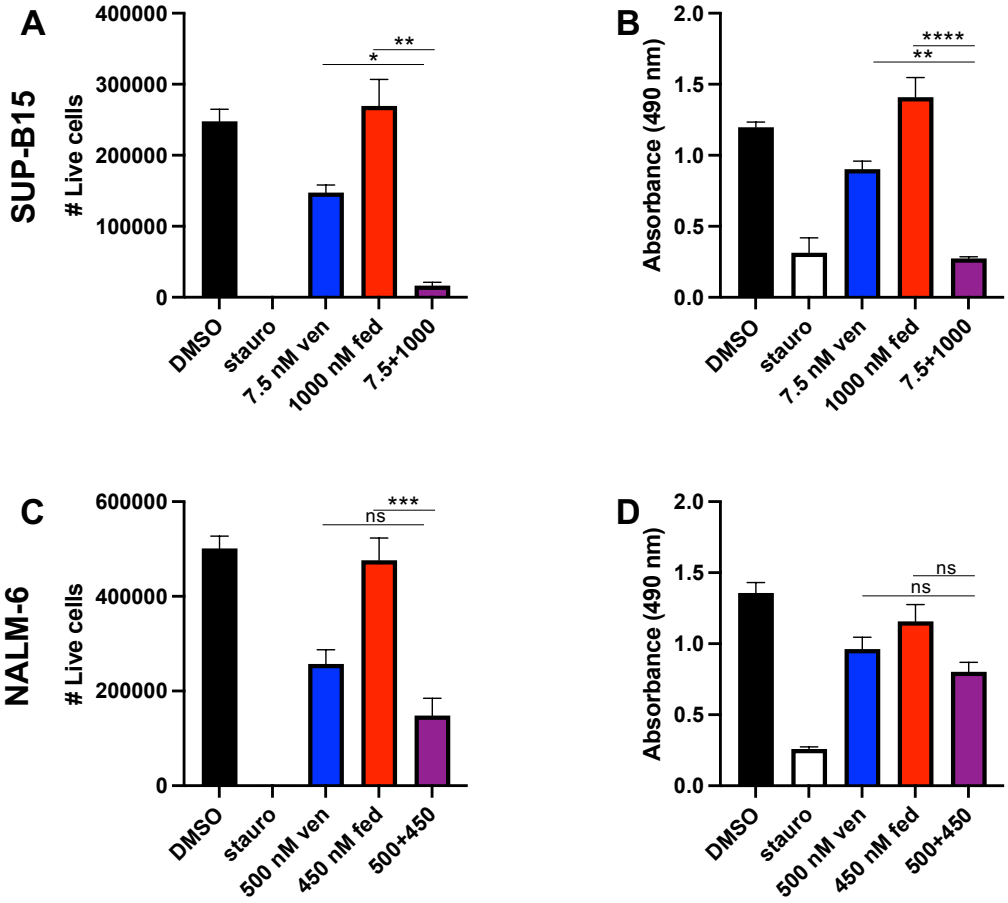
A
SUP-B15
Live cells
400000
300000
200000
100000
0
*
**
DMSO
stauro
7.5 nM ven
1000 nM fed
7.5+1000
B
Absorbance (490 nm)
2.0
1.5
1.0
0.5
0.0
****
**
DMSO
stauro
7.5 nM ven
1000 nM fed
7.5+1000
C
NALM-6
Live cells
600000
400000
200000
0
ns
***
DMSO
stauro
500 nM ven
450 nM fed
500+450
D
Absorbance (490 nm)
2.0
1.5
1.0
0.5
0.0
ns
ns
DMSO
stauro
500 nM ven
450 nM fed
500+450

Figure 2

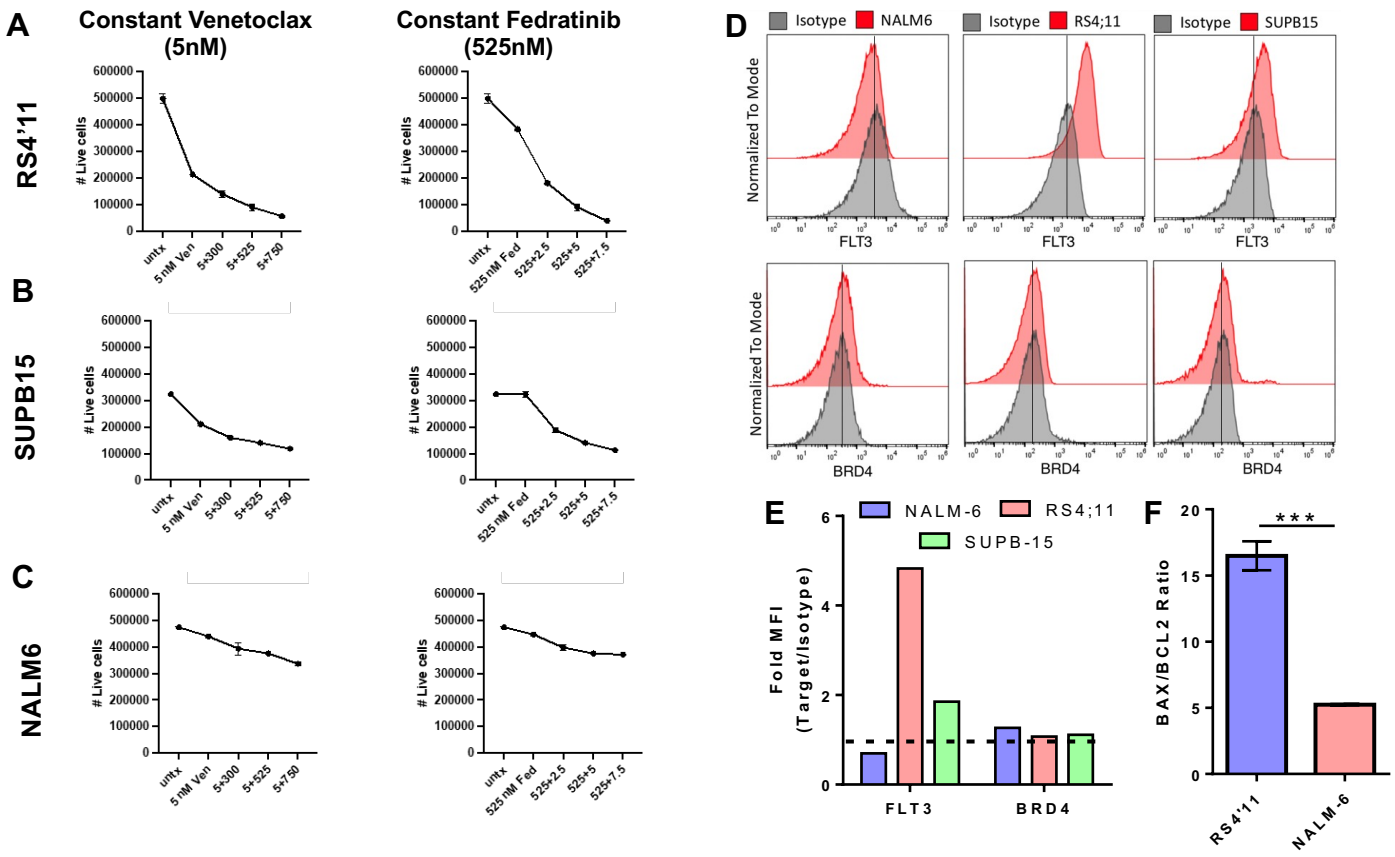
A
Constant Venetoclax (5nM)
Constant Fedratinib (525nM)
RS4'11
B
SUPB15
C
NALM6
Live cells
untx
5 nM Ven
5+300
5+525
5+750
525 nM Fed
525+2.5
525+5
525+7.5
D
Isotype
NALM6
RS4;11
SUPB15
Normalized To Mode
FLT3
BRD4
E
NALM-6
RS4;11
SUPB-15
Fold MFI (Target/Isotype)
F
***
BAX/BCL2 Ratio
RS4'11
NALM-6

Figure 3

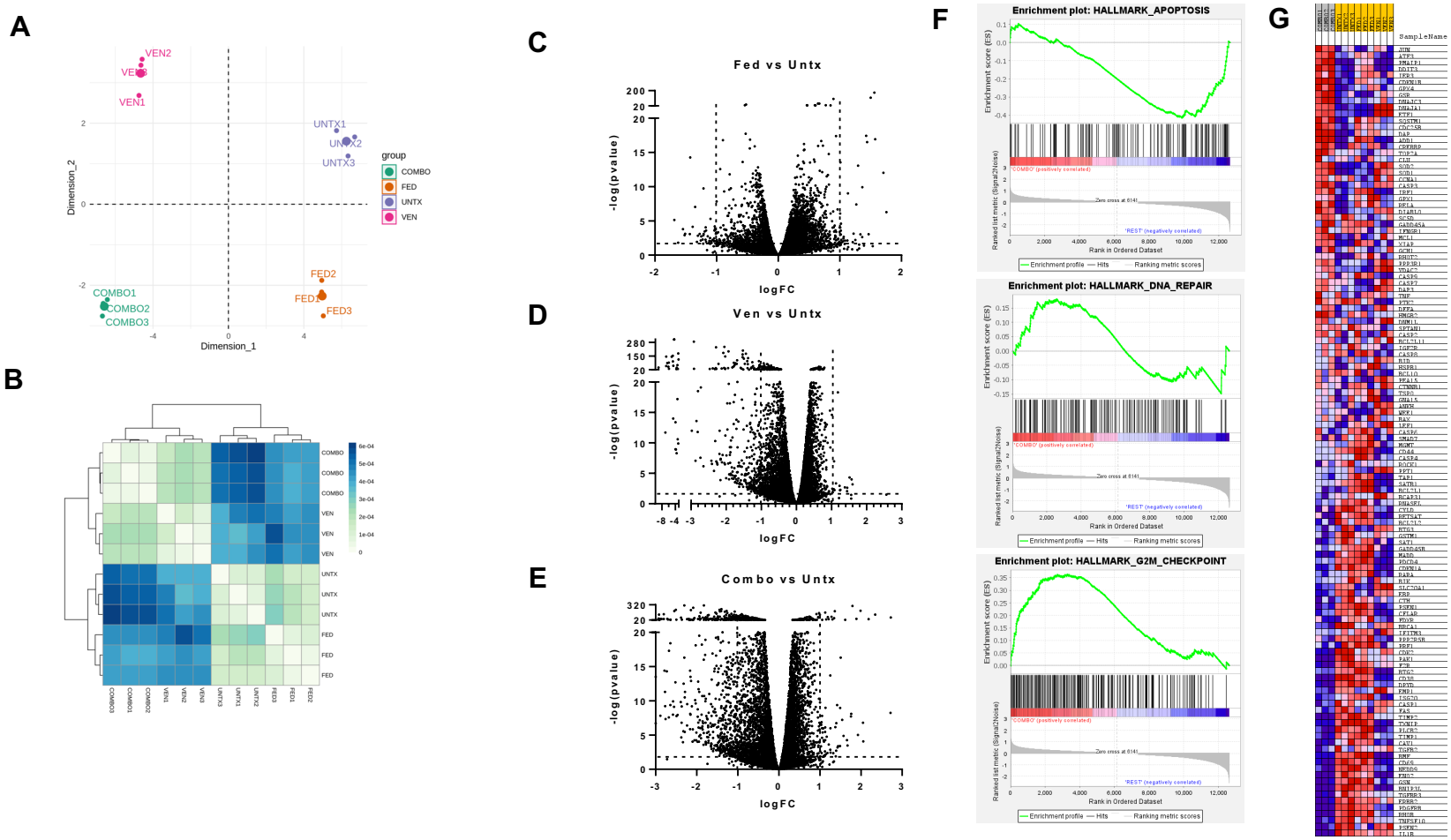
A
VEN2
VEN3
VEN1
UNTX1
UNTX2
UNTX3
FED2
FED1
FED3
COMBO1
COMBO2
COMBO3
Dimension_1
Dimension_2
group
COMBO
FED
UNTX
VEN
B
C
Fed vs Untx
-log(pvalue)
logFC
D
Ven vs Untx
E
Combo vs Untx
F
Enrichment plot: HALLMARK_APOPTOSIS
Enrichment plot: HALLMARK_DNA_REPAIR
Enrichment plot: HALLMARK_G2M_CHECKPOINT
Enrichment score (ES)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
G
SampleName

Figure 4

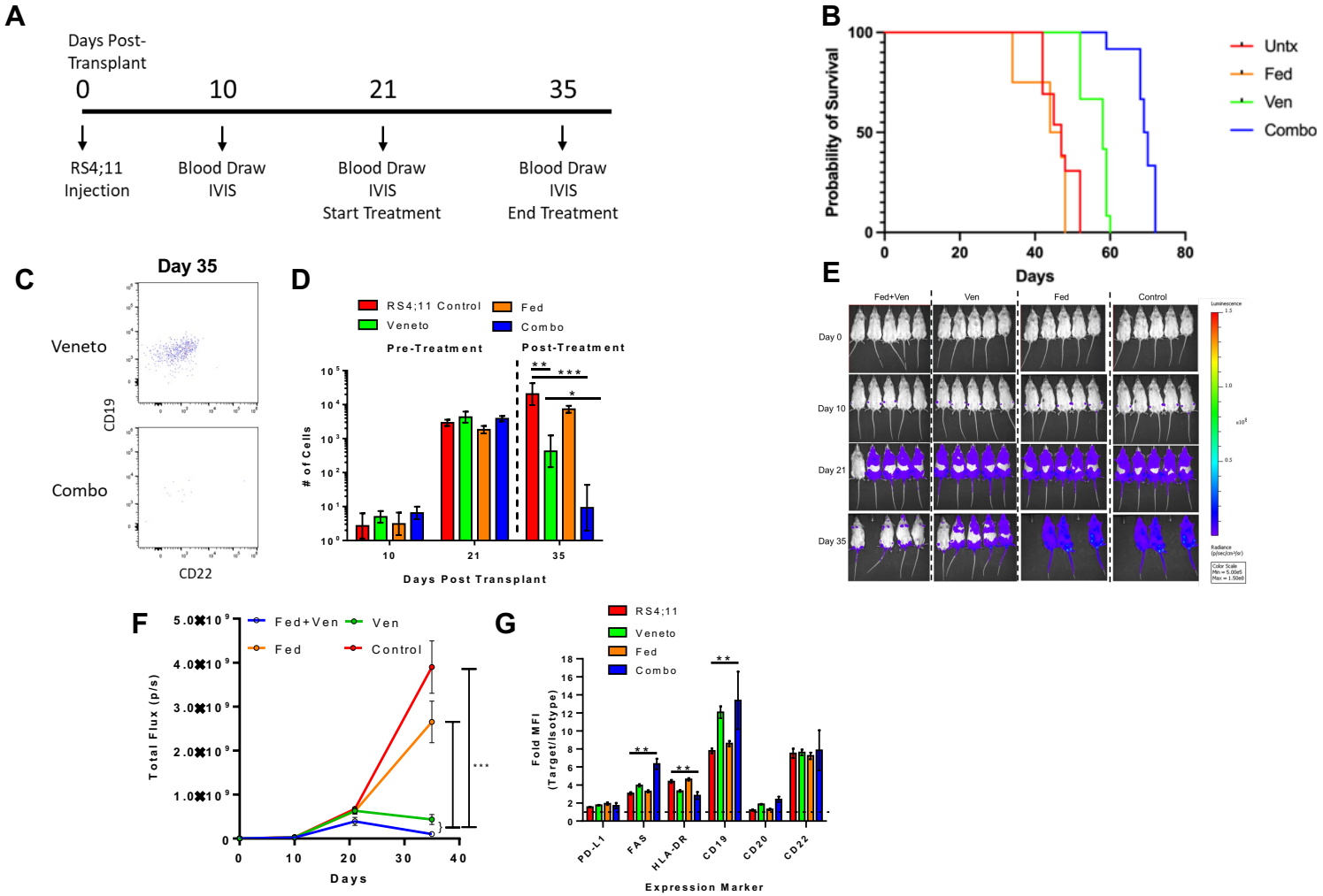
A
Days Post-Transplant
0
10
21
35
RS4;11 Injection
Blood Draw IVIS
Blood Draw IVIS Start Treatment
Blood Draw IVIS End Treatment
B
Probability of Survival
Days
Untx
Fed
Ven
Combo
C
Day 35
Veneto
Combo
CD19
CD22
D
RS4;11 Control
Veneto
Fed
Combo
Pre-Treatment
Post-Treatment
# of Cells
Days Post Transplant
E
Fed+Ven
Ven
Fed
Control
Day 0
Day 10
Day 21
Day 35
F
Fed+Ven
Ven
Fed
Control
Total Flux (p/s)
Days
G
RS4;11
Veneto
Fed
Combo
Fold MFI (Target/Isotype)
PD-L1
FAS
HLA-DR
CD19
CD20
CD22
Expression Marker

Figure 5