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ORIGINAL ARTICLE

Suppression of multiple anti-apoptotic BCL2 family proteins recapitulates the effects of JAK2 inhibitors in JAK2V617F driven myeloproliferative neoplasms

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Abstract

Several lines of research suggest that Bcl-xL-mediated anti-apoptotic effects may contribute to the pathogenesis of myeloproliferative neoplasms driven by JAK2V617F and serve as therapeutic target. Here, we used a knock-in JAK2V617F mouse model and confirmed that Bcl-xL was overexpressed in erythroid progenitors. The myeloproliferative neoplasm (MPN)-induced phenotype in the peripheral blood by conditional knock-in of JAK2V617F was abrogated by conditional knockout of *Bcl2l1*, which presented anemia and thrombocytopenia independently of JAK2 mutation status. *Mx1-Cre Jak2V617*$^{W/VF}$*/Bcl2l1*$^{f/f}$ mice presented persistent splenomegaly as a result of extramedullary hematopoiesis and pro-apoptotic stimuli in terminally differentiated erythroid progenitors. The pan-BH3 mimetic inhibitor obatoclax showed superior cytotoxicity in JAK2V617F cell models, and reduced clonogenic capacity in ex vivo

Abbreviations: AML, acute myeloid leukemia; APC, allophycocyanin; BFU-E, erythroid colony-forming unit; BIO, biotin; CFU-G, granulocyte colony-forming unit; CFU-GEMM, Colony-forming unit of granulocytes, erythrocytes, monocytes, and megakaryocytes; CFU-GM, granulocyte/monocyte colony-forming unit; CFU-M, monocyte colony-forming unit; ET, essential thrombocytosis; Ht, hematocrit; MPN, myeloproliferative neoplasms; PB, pacific blue; PE, phycoerythrin; pIpC, polyinosinic:polycytidylic acid; PLT, platelet; PMF, primary myelofibrosis; PV, polycythemia vera; WBC, white blood cell.

Hisashi Takei and Juan Luiz Coelho-Silva contributed equally to this work.

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assay using Vav-Cre Jak2V617F bone marrow cells. Both ruxolitinib and obatoclax significantly reduced spleen weights in a murine Jak2V617F MPN model but did not show additive effect. The tumor burden reduction was observed with either ruxolitinib or obatoclax in terminal differentiation stage neoplastic cells but not in myeloid-erythroid precursors. Therefore, disrupting the BCL2 balance is not sufficient to treat MPN at the stem cell level, but it is certainly an additional option for controlling the critical myeloid expansion of the disease.

KEYWORDS
apoptosis, BCL2 family proteins, JAK2V617F, mouse models, myeloproliferative neoplasms

1 | INTRODUCTION

The BCR-ABL1-negative classical myeloproliferative neoplasms (MPN) are a group of heterogeneous hematologic malignancies that involve a clonal proliferation of hematopoietic stem cells characterized by excessive production of terminally differentiated blood cells that are fully functional. They are classified in 3 main entities known as PV, ET and PMF, that differ clinically by a marked erythrocytosis in PV, thrombocytosis in ET and extensive bone marrow (BM) fibrosis and extramedullary hematopoiesis in PMF. In common, they share initiating mutations such as JAK2V617F, CALR, and MPL, as well as BM panmyelosis, variable degrees of fibrosis, extramedullary hematopoiesis, and increased risk for thrombosis, hemorrhage, and progression to AML.

JAK2 mutations are present in more than 95% of PV patients and more than 50% of ET and PMF patients. However, JAK2 inhibition by the FDA-approved drug ruxolitinib improves constitutional symptoms and splenomegaly but does not significantly reduce BM fibrosis or the mutant allele burden in patients. The disappointing results of JAK2 inhibition in its ability to eradicate JAK2-mutated initiating cells prompts us to search other potential targets. These may include target genes that can be activated or repressed by STAT3 and STAT5, which have both been shown to play key roles in cellular transformation and regulate the expression of genes that control processes such as proliferation, apoptosis, and angiogenesis.

Among these, the BCL2 family proteins have recently been pointed out as important targets in myeloid neoplasms. The BCL2 family proteins regulate the intrinsic mitochondrial apoptosis pathway and are comprised of 3 subgroups based on structure and function: the pro-apoptotic BAX and BAK-like proteins, the anti-apoptotic BCL2 proteins, and the BH3-only proteins. Anti-apoptotic BCL2 proteins such as BCL2 or BCL-XL bind to and inhibit the pro-apoptotic BAX or BAK, which can permeabilize the mitochondrial outer membrane and initiate caspase activation.[1] BCL-XL (also known as BCL2L1) has been suggested to play a critical role in the pathogenesis of PV over the past 2 decades. BCL-XL is transcriptionally regulated by STAT3/5 downstream of JAK2 and overexpressed in erythroid cells from patients with PV.[2] STAT5 and BCL-XL can induce erythroid colony formation from erythroid precursors in the absence of erythropoietin.[3] Furthermore, JAK2 V617F contributes to inhibition of BCL-XL deamidation induced by DNA damage, which reduces apoptosis.[4] In contrast, downregulation of BCL-XL by either JAK2 inhibitors or RNA interference leads to apoptosis in *JAK2* mutant cells.[5] In addition to BCL-XL, we and others have shown that BIM, a BH3-only protein is induced by JAK inhibition and plays an important role in cell killing.[6,7] However, the role of other BCL2 family proteins is not well described.

In this study, we sought to determine the role of BCL2 family proteins in JAK inhibition using cell lines and mouse models. We demonstrated that obatoclax, a BH3 mimetics that broadly inhibits anti-apoptotic BCL2 family proteins, was as effective as ruxolitinib in reducing tumor burden. Our results underline the complexity of apoptotic machinery in the pathogenesis and treatment of MPN induced by JAK2 V617F.

2 | MATERIALS AND METHODS

2.1 | Mice

Heterozygous wild-type/flox mice harboring the silent *Jak2*V617F mutation (*Jak2*$^{w/VF}$) were previously generated[8] and bred to either Vav-Cre[9] or Mx1-Cre[10] transgenics, also on the C57BL/6 background (gift from Dr. Daniel G. Tenen at Harvard Medical School). Exposure to Cre recombinase resulted in inversion of the mutant exon followed by an excision reaction that removes the wild-type (w) exon, 1 *loxP* site, and 1 *loxP511* site, fixing the *Jak2*V617F inversion in place. In the Vav-Cre model *Jak2* mutation was specifically expressed in the hematopoietic system and endothelial cells, therefore resulting in a lethal myeloproliferative neoplasm as in the original model. In the Mx1-Cre model, the Mx dynamin-like GTPase 1 (Mx1) promoter is activated in an interferon-dependent manner following polyinosinic:polycytidylic acid (pIpC) injection. *Bcl-xl* knockout mice were previously established[11] and resulted from the removal of exons 1 and 2 mediated by Cre recombinase (gift from Dr. Lothar Hennighausen at National Institution of Diabetes and Digestive and Kidney Diseases). All mice were housed in a sterile barrier facility.

2.2 | Peripheral blood analysis

Blood was collected by submandibular sampling into EDTA-coated containers and used for complete blood counts on a Hemavet 950 analyzer (Drew Scientific) or for chimerism detection by flow cytometry.

2.3 | Flow cytometry

For progenitor analysis, single-cell suspensions from murine BM or spleen were analyzed by flow cytometry using the following monoclonal antibodies conjugated to biotin (BIO), fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-CY5, PE-CY7, PB, allophycocyanin (APC) or APC-Cy7 obtained from BioLegend or eBioscience: CD19 (MB19-1), B220 (RA3-6B2), CD4 (RM4-5), CD8 (53-6.7), Gr1 (RB6-8C5), CD11b (M1/70), and streptavidin for lineage exclusion; Sca-1 (D7), c-Kit (2B8), CD34 (RAM34), CD41 (MWReg30), CD150 (TC15-12F12.2), FcγRII/III (93), Endoglin (MJ7/18), TER119 (TER-119), CD71 (OKT-9) for myeloerythroid progenitor subpopulations; CD45.1 (A20), CD45.2 (104) as well as differentiation markers CD3 (17A2), B220 (RA3-6B2), Gr1 (RB6-8C5), CD11b (M1/70) for engraftment analysis. Stained cells were analyzed in a LSRII flow cytometer and sorted using a FACSAria II instrument (BD Biosciences). Viable cells were identified by DAPI exclusion. Diva (BD BioSciences) and FlowJo (Tree Star) software were used for data acquisition and analysis, respectively.

2.4 | Western blot analysis

Equal amounts of protein were used as total extracts, followed by SDS-PAGE, Western blot analysis was performed with the indicated antibodies and imaging was carried out using the SuperSignal™ West Dura Extended Duration Substrate System (Thermo Fisher Scientific, San Jose, CA, USA) and G:BOX Chemi XX6 gel doc systems (Syngene, Cambridge, UK). Antibodies against STAT5 (#25656), BCL-XL (#2764), BIM (#2933), PARP1 (#9542), γH2AX (#9718), β-actin (#4970), and α-tubulin (#2144) were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.5 | Histopathology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with H&E.

2.6 | BCL2-related gene expression analysis

The expression of 19 members of the BCL2 gene family in samples from naïve SET2 cells (GSM1817344, GSM1817345, and GSM1817346) and ruxolitinib-treated SET2 cells (GSM1817332, GSM1817333, and GSM1817334) was investigated using RNA-seq data from Meyer et al[12] (https://www.ncbi.nlm.nih.gov/geo; GEO accession GSE69827). Gene expression was indicated as fold change of the mean of normalized counts of naïve SET2 cells, which was set as 1, and illustrated using multiple experiment viewer (MeV) 4.9.0 software.[13] The gene expression levels of Bcl2 family genes were assessed in sorted murine subpopulations KSL ($Lin^-Sca1^+cKit^{hi}$) and myeloid-erythroid progenitors (MEP; $Lin^-Sca1^-cKit^{hi}FcγRII/III^-CD34^{-/dim}$). Total RNA was obtained using the RNeasy Plus Mini Kit (Qiagen) and samples with RNA integrity were taken for mRNA transcription; real-time PCR was performed using oligonucleotides designed (Integrated DNA Technologies) for the following gene references: *Bcl2*, NM_009741.3; *Bcl2l1*, NM_009743.4; *Mcl-1*, NM_008562.3; *Bim*, NM_207680.2; and *Bax*, NM_007527.3.

2.7 | Cell culture and inhibitors

The HEL cell line was obtained from the ATCC (Philadelphia, PA, USA). SET2 cells were kindly provided by Professor Fabíola Attié de Castro (School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil). SET2 and HEL cells harboring the JAK2V617F mutation were tested and authenticated by short tandem repeat matching analysis using the PowerPlex® 16 HS system (Promega, Madison, WI, USA) and the ABI 3500 Sequence Detector System (Life Technologies, Foster City, CA, USA). Cell culture conditions were performed in accordance with the recommendations of the ATCC and DSMZ. All cell lines were mycoplasma free. Ruxolitinib was obtained from InvivoGen (San Diego, CA, USA). Venetoclax (ABT-199) was obtained from TargetMol (Wellesley Hills, MA, USA). Navitoclax (ABT-263) was obtained from LC laboratories (Woburn, MA, USA). Obatoclax was obtained from Chemietek (Indianapolis, IN, USA).

2.8 | Cell viability assay

Cell viability was measured by MTT assay. SET2 (4×10^4 cells/well) and HEL cells (2×10^4 cells/well) were cultured in a 96-well plate in RPMI medium containing 10% or 20% FBS, respectively, in the presence of increasing concentrations of venetoclax (ABT-199), navitoclax (ABT-263), or obatoclax for 72 h. DMSO was used as a negative control. IC_{50} values were calculated using nonlinear regression analysis in GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA, USA).

2.9 | Colony-forming unit assays

Bone marrow mononuclear cells (BMMC) were isolated from $Jak2^{w/w}$ (n = 3) and $Jak2^{w/VF}$ (n = 3) mice by Ficoll-gradient centrifugation. In total, 1×10^5 cells were plated onto methocult GF3434 (StemCell Technologies Inc, Vancouver, Canada) in the presence

or not of ruxolitinib (300 nmol/L) and/or obatoclax (250 nmol/L). Colonies were counted under an inverted microscope after 10 d of culture and classified according to their morphology into granulocyte-monocyte colony-forming unit (CFU-GM), granulocyte colony-forming unit (CFU-G), monocyte colony-forming unit (CFU-M), erythroid colony-forming unit (BFU-E) or colony-forming unit of granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM).

2.10 | Bone marrow transplantation

Whole BM cells from *Vav-Cre/Jak2*$^{w/w}$ or *Vav-Cre/Jak2*$^{w/VF}$ mice were injected through the retro-orbital venous sinus into lethally irradiated recipients (wild-type C57BL/6 CD45.1 mouse strain, n = 4/group). For engraftment analysis, cells were stained with anti-CD45.1 and CD45.2 antibodies to distinguish donor-derived cells from the host cells, as well as with lineage-specific antibodies Mac1, Gr1, B220, and CD3 to identify myeloid, B cell and T cell lineages, respectively. Treatment with vehicle (5% dimethylacetamide 0.5% methocellulose), Jak2 inhibitor (JAK2 I, ruxolitinib 45 mg/kg/d i.p.)[14] or BCL2 inhibitor (BCL2 I, the BH3 mimetic obatoclax 3 mg/kg/d i.p.)[15] was started 4 wk after transplant (when chimerism was greater than 80% and hosts presented Ht > 70%). At 8 wk after transplant, mice were bled and euthanized to assess disease burden.

2.11 | Statistical analysis

All experimental data were expressed as mean and standard deviation, and differences were evaluated by Mann-Whitney test (Figure 1), unpaired two-tailed Student *T* test (Figures 2 and 4), or ANOVA with Bonferroni post-test using an α value of 5% (Figure 5). All statistical calculations were made using GraphPad Prism 7 software.

3 | RESULTS

3.1 | Heterozygous *Jak2V617F Vav-Cre*-driven expressing animals develop a lethal MPN, with erythroid expansion and Bcl-2 deregulation

Differently from the original model, *Jak2*$^{w/VF}$ mice were crossed to *Vav-Cre* instead of the *E2A-Cre* transgenic mice to obtain the *Jak2V617F* germline expression specifically at the hematopoietic compartment driven by the *Vav* promoter. Therefore, MPN had to be confirmed and was characterized by elevated Ht and PLT counts as well as splenomegaly due to extramedullary hematopoiesis (all $P < .01$), as shown in Figure 1A, resembling the original model, with a 100% penetrance. To confirm the myeloproliferative phenotype, we further performed a detailed flow cytometry analysis of the BM myeloid precursors[16,17] and found increased absolute numbers of both Lineage negative (Lin^-)$cKit^{high}Sca\text{-}1^-CD150^+CD41^-Endoglin^-$ pre-MegE (pre-megakaryocyte-erythrocyte) cells (with erythroid and megakaryocytic potential) and $Lin^-cKit^{high}Sca\text{-}1^-CD150^+CD41^+$MkP (late megakaryocyte progenitors) in *Jak2*$^{w/VF}$ mice compared with the controls. Erythroid expansion was also confirmed by the higher numbers of $Lin^-cKit^{high}Sca\text{-}1^-CD150^+CD41^-Endoglin^+$ pre-CFU cells and their terminal $Lin^-cKit^{high}Sca\text{-}1^-CD150^-CD41^-Endoglin^+$ CFU-E ($CD71^+$ $Ter\text{-}119^-$) and pro-E ($CD71^+Ter\text{-}119^+$) subpopulations. Of note, no significant quantitative differences in KSL ($Lin^-cKit^{high}Sca\text{-}1^+$), GMP ($Lin^-$ $Kit^{high}Sca\text{-}1^-Fc\gamma RII/III^{high}CD34^+$) or CMP ($Lin^-cKit^{high}Sca\text{-}1^-Fc\gamma RII/III^{high}CD34^+$) numbers were observed between *Jak2*$^{w/w}$ and *Jak2*$^{w/VF}$ mice (Figure S1).

A prominent expansion of early ($Lin^-CD71^+Ter119^+$) rather than late ($Lin^-CD71^-Ter119^+$) erythroid cells was confirmed in the spleen of *Jak2*$^{w/VF}$ mice compared with the controls, demonstrating extramedullary hematopoiesis (Figure 1B). To study apoptosis resistance in this model, cellular extracts were obtained from the spleens and analyzed by western blotting. Overexpression of STAT-5 and Bcl-xL, and Bim downregulation in *Jak2*$^{w/VF}$ mice compared with *Jak2*$^{w/w}$ controls was observed, indicating greater abundance in erythroid progenitors in the spleen in *Jak2*$^{w/VF}$ mice (Figure 1C).

3.2 | Bcl-xL expression partially modifies the murine *Jak2*V617F MPN phenotype

Based on our results and previous reports demonstrating that BCL-XL plays a pivotal role in pathogenesis in PV, we sought to determine whether genetic deletion of *Bcl2l1*, which encodes Bcl-x, would reverse the Jak2V617F hematologic phenotypes including increased erythropoiesis and thrombocytosis. Conditional deletion of *Bcl2l1* reportedly leads to severe anemia and splenomegaly.[18] Further studies demonstrated that Bcl-xL is only required for apoptosis prevention during late-stage erythropoiesis.[19,20] These observations prompted to us to ask: (a) whether expression of Jak2V617F would prevent apoptosis during late-stage erythropoiesis and megakaryopoiesis in conditional *Bcl2l1* knockout mice; and (b) whether overexpression of Bcl-xL induced by Jak2V617F would contribute to erythroid and megakaryocytic hyperplasia and its deletion would revert Jak2V617F myeloproliferation. To this end, we crossed *Jak2*$^{w/VF}$ mice with *Mx1-Cre* mice and *Bcl2l1* conditional mice.[11] In the resultant *Mx1-Cre/Jak2*$^{w/VF}$/*Bcl2l1*$^{f/f}$ mice, expression of Jak2V617 and deletion of *Bcl2l1* were simultaneously induced by pIpC injection in hematopoietic cells.

An increase in Ht and numbers of WBC and PLT counts were observed 4 wk after pIpC injections in *Mx1-Cre/Jak2*$^{w/VF}$/*Bcl2l1*$^{w/w}$ mice compared with *Mx1-Cre/Jak2*$^{w/w}$/*Bcl2l1*$^{w/w}$ control mice (Figure 2A: yellow vs. blue lines) as seen in *Vav-Cre/Jak2*$^{w/VF}$ mice (Figure 1A). In contrast with *Vav-Cre/Jak2*$^{w/VF}$ mice (Figure 1A), an increase in WBC numbers was observed in *Mx1-Cre/Jak2*$^{w/VF}$/*Bcl2l1*$^{w/w}$ mice

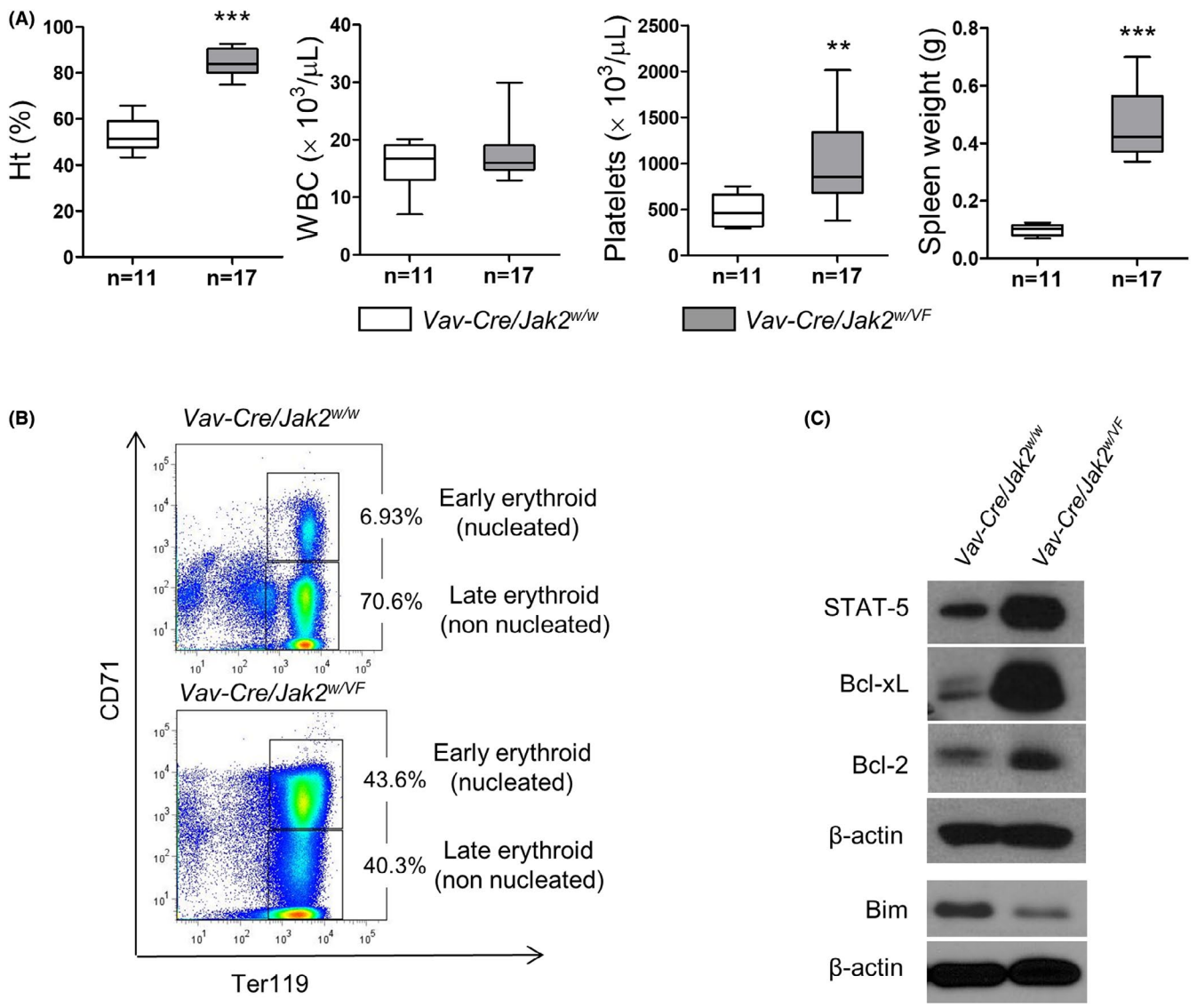


FIGURE 1 Characterization of myeloid proliferation in the *Vav-Cre/Jak2V617F* murine model. The heterozygous *Jak2V617F* expressing animals (*Jak2*$^{w/VF}$) develop a lethal myeloproliferative neoplasm characterized by elevated Ht, WBC, and splenomegaly due to extramedullary hematopoiesis (A), and a prominent expansion of early (Lin$^-$CD71$^+$Ter119$^+$) erythroid cells in whole cell extracts from the spleen (B, flow cytometry representative analysis), associated with upregulation of STAT-5, Bcl-xL, Bcl-2 and downregulation of Bim (C). Asterisks indicate *P*-value as follows: $^{**}P < .01$, $^{***}P < .001$

(Figure 2A: middle panel). Similarly, the activation of Jak2V617F mutation in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/f}$ increased peripheral blood counts (Figure 2A: orange vs. purple lines). The *Mx1-Cre/Jak2*$^{w/w}$*/Bcl2l1*$^{f/f}$ mice developed anemia and presented much lower WBC and PLT counts (Figure 2A: green vs. blue lines). Of note, expression of the *Jak2*V617F mutation was not able to overcome cytopenia caused by the *Bcl2l1* knockout (Figure 2A: green vs. red lines). Similarly, a decrease in Ht, WBC and PLT counts observed in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ mice compared with *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/w}$ mice, indicated that the *Bcl2l1* deletion resulted in MPN phenotype reversal when anti-apoptotic control mediated by this protein was lost (Figure 2A: red vs. yellow lines).

Next, to determine whether the decrease in Ht, WBC and PLT counts mediated by Bcl-xL loss in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ was due to suppression of erythropoiesis/megakaryopoiesis or lack of maturation of these cells, we analyzed BM progenitor cells in mice treated with pIpC. Compared with non-induced controls (*Jak2*$^{w/VF}$*/Bcl2l1*$^{w/w}$ mice), an increase in MEP cells was observed in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/w}$ mice; however, no decrease in MEP was observed in *Mx1-Cre/Jak2*$^{w/w}$*/Bcl2l1*$^{f/f}$. Although the Jak2 mutation was not able to increase peripheral blood counts in *Bcl2l1*-deficient mice, increased MEP was seen in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ mice compared with *Mx1-Cre/Jak2*$^{w/w}$*/Bcl2l1*$^{f/f}$ (Figure S2). Consistent with these observations, hypercellular BMs with an increase in erythrocytes and PLT were observed in both *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/w}$ and *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ mice, as well as *Mx1-Cre/Jak2*$^{w/w}$*/Bcl2l1*$^{f/f}$ mice (Figure 2B). Altogether, these results suggested that the Jak2V617F mutation is able to induce

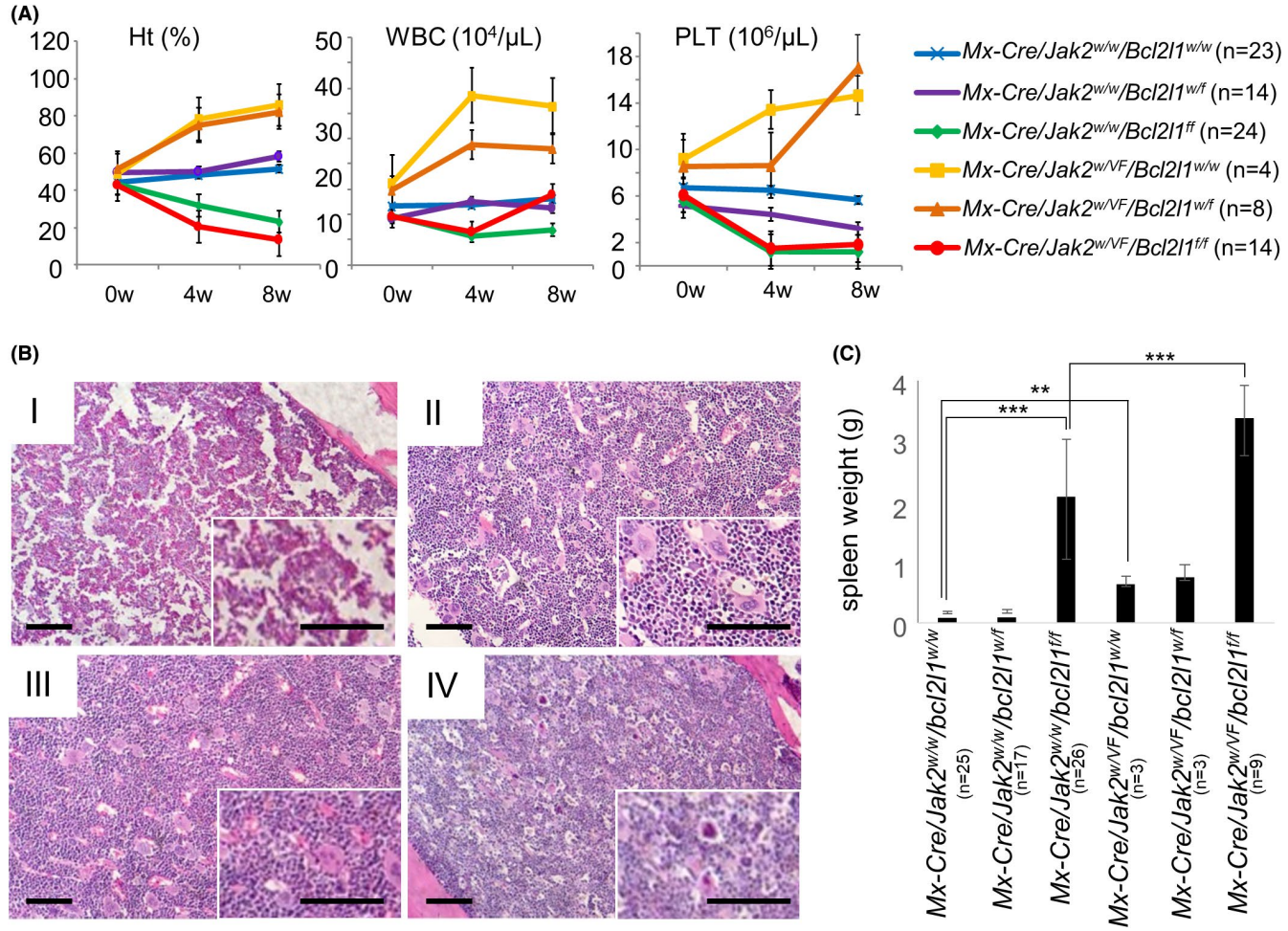


FIGURE 2 Bcl-xL abrogation partially rescues the MPN phenotype in a Jak2V617F-mutated mice. A, Percentages of blood Ht and absolute cell numbers for WBC) and PLT in the peripheral blood of mice are indicated. The bars represent mean ± SD values. B, H&E staining of bone marrow (BM) isolated from: I *JAK2*$^{w/VF}$/*Bcl2l1*$^{w/f}$; II *Mx1-Cre/JAK2*$^{w/VF}$/*Bcl2l1*$^{w/f}$; III *Mx1-Cre/JAK2*$^{w/VF}$/*Bcl2l1*$^{w/w}$; IV: *Mx1-Cre/JAK2*$^{w/VF}$/*Bcl2l1*$^{f/f}$. Scale bars, 100 μm. C, Spleen weight isolated from mice indicated after 8 wk of pIpC injection. The bars represent mean ± SD values. Asterisks indicate *P*-value as follows: $^{**}P < .01$, $^{***}P < .001$

erythroid and megakaryocytic hyperplasias, independently of Bcl-x, resulting in an increase in earlier myeloid progenitors but is unable to overcome apoptosis during late-stage erythropoiesis and megakaryopoiesis induced by Bcl-x loss.

As previously described in several conditional *Bcl2l1* mice models,[18-20] deletion of *Bcl2l1* alone led to splenomegaly. Mild splenomegaly was observed in *Mx1-Cre/Jak2*$^{w/VF}$/*Bcl2l1*$^{w/w}$ and *Mx1-Cre/Jak2*$^{w/VF}$/*Bcl2l1*$^{w/f}$ mice. *Mx1-Cre/Jak2*$^{w/VF}$/*Bcl2l1*$^{f/f}$ mice had significantly larger spleens than *Mx1-Cre/Jak2*$^{w/w}$/*Bcl2l1*$^{f/f}$ (Figure 2C), suggesting that Jak2V617F had an additive effect on splenomegaly and may enhance extramedullary erythropoiesis.

Taken together, these results suggested that expression of Jak2V617F is not involved in Bcl-xL maturation of late-stage erythropoiesis but reinforced the hypothesis that Bcl-xL contributes to erythroid and megakaryocytic hyperplasia induced by Jak2V617F as its abrogation partially reverted the MPN phenotype by reducing blood counts, although pro-apoptotic compensatory splenomegaly persists as a major consequence of Bcl-xL deficiency.

3.3 | Inhibition of multiple anti-apoptotic members of the BCL2 family is necessary to effectively reduce cell viability in JAK2V617F-positive cell lines

Given the partial reversion of MPN myeloid expansion by *Bcl-xL* deletion, we next hypothesized that other BCL2 family member(s) played a role in this process. We first used SET2 cells, which express JAK2V617F to evaluate changes in expression of the BCL2 family genes upon inhibition of the JAK2/STAT pathway by ruxolitinib. Genes of the anti-apoptotic members, in particular *BCL2*, *BCL2L1*, *MCL1*, and *BCL2L10*, were downregulated, while the pro-apoptotic members, in particular *BAX*, *BAD*, *BMF*, and *BNIP3L*, were upregulated, indicating that JAK2 inhibition resulted in imbalance between anti- and pro-apoptotic members of the BCL2 family (Figure 3A). Next, the effects of selective BCL2 (venetoclax), BCL2/BCL-XL (navitoclax) or pan anti-apoptotic BCL2 (obatoclax) inhibitors were investigated in SET2 and HEL cells,

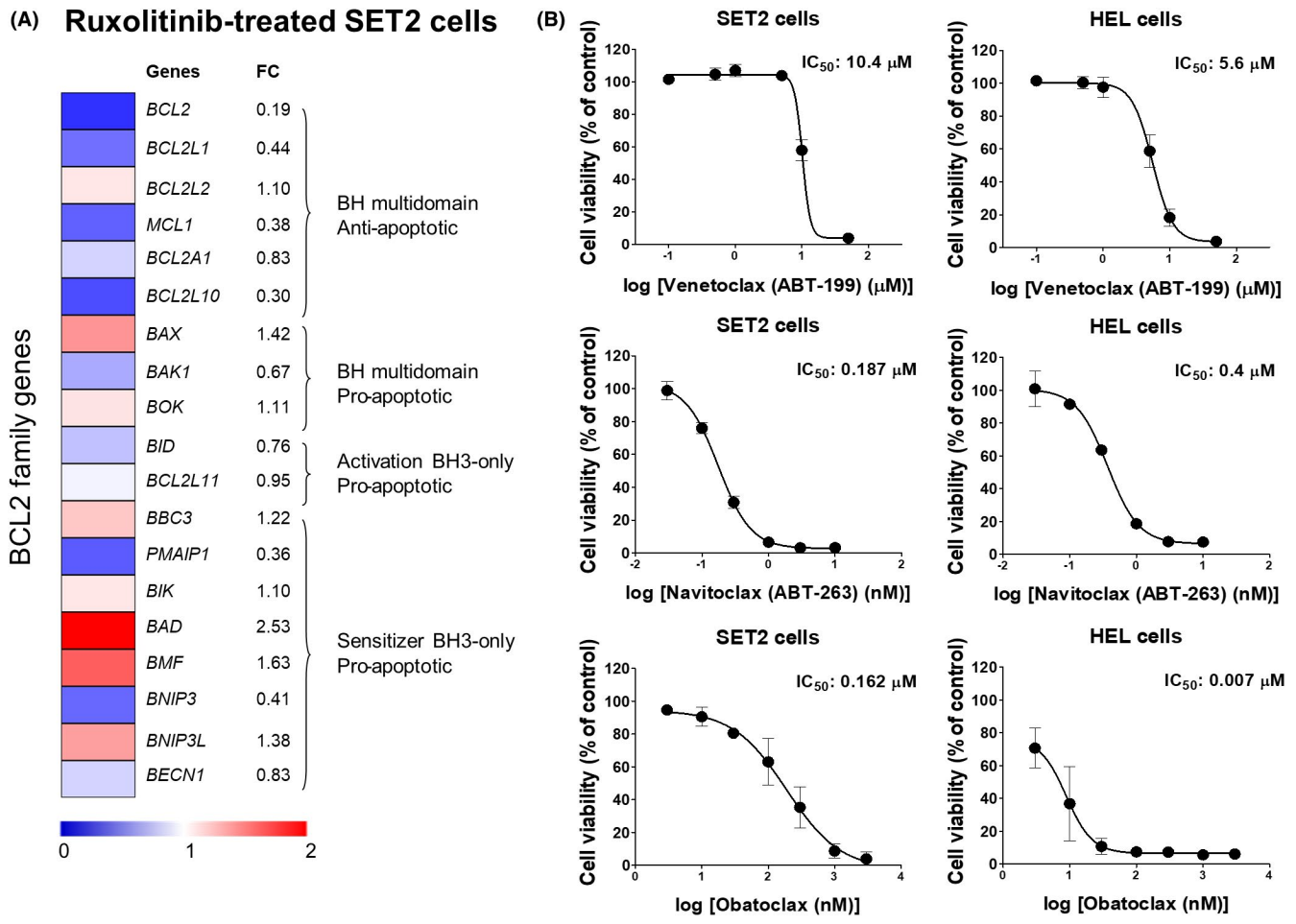


FIGURE 3 Inhibition of multiple anti-apoptotic proteins of the BCL2 family reduces cell viability more effectively. A, Heatmap containing BCL2 family genes from RNA-seq analysis of SET2 cells upon ruxolitinib treatment (GEO accession GSE69827). Gene expression was expressed as fold change of the mean of normalized counts of naïve SET2 cells, which was set as 1 and the mean of fold change obtained from 3 experimental replicates is indicated. B, Dose-response cytotoxicity for SET2 and HEL cells treated with increasing concentrations of venetoclax (ABT-199), navitoclax (ABT-263), or obatoclax for 72 h. Values are expressed as the percentage (mean ± SD) of viable cells relative to vehicle-treated controls. The IC_{50} values are indicated. C, Western blot analysis of PARP1 (total and cleaved) and γH2AX in total cell extracts from SET2 and HEL cells treated with vehicle or obatoclax (3, 30 or 300 nmol/L); membranes were reprobed with the antibody for the detection of the respective total protein or α-tubulin, and revealed with the SuperSignal™ West Dura Extended Duration Substrate system using the G:BOX Chemi XX6 gel doc system

another JAK2V617F-positive cell line. Among the tested inhibitors, obatoclax displayed the highest potency, indicating that the inhibition of multiple anti-apoptotic BCL2 family members is necessary to effectively reduce cell viability in cellular models with constitutive activation of the JAK2/STAT pathway (Figure 3B). The induction of apoptosis by obatoclax was confirmed by the induction of cleaved PARP1 and γH2AX in SET2 and HEL cells (Figure 3C).

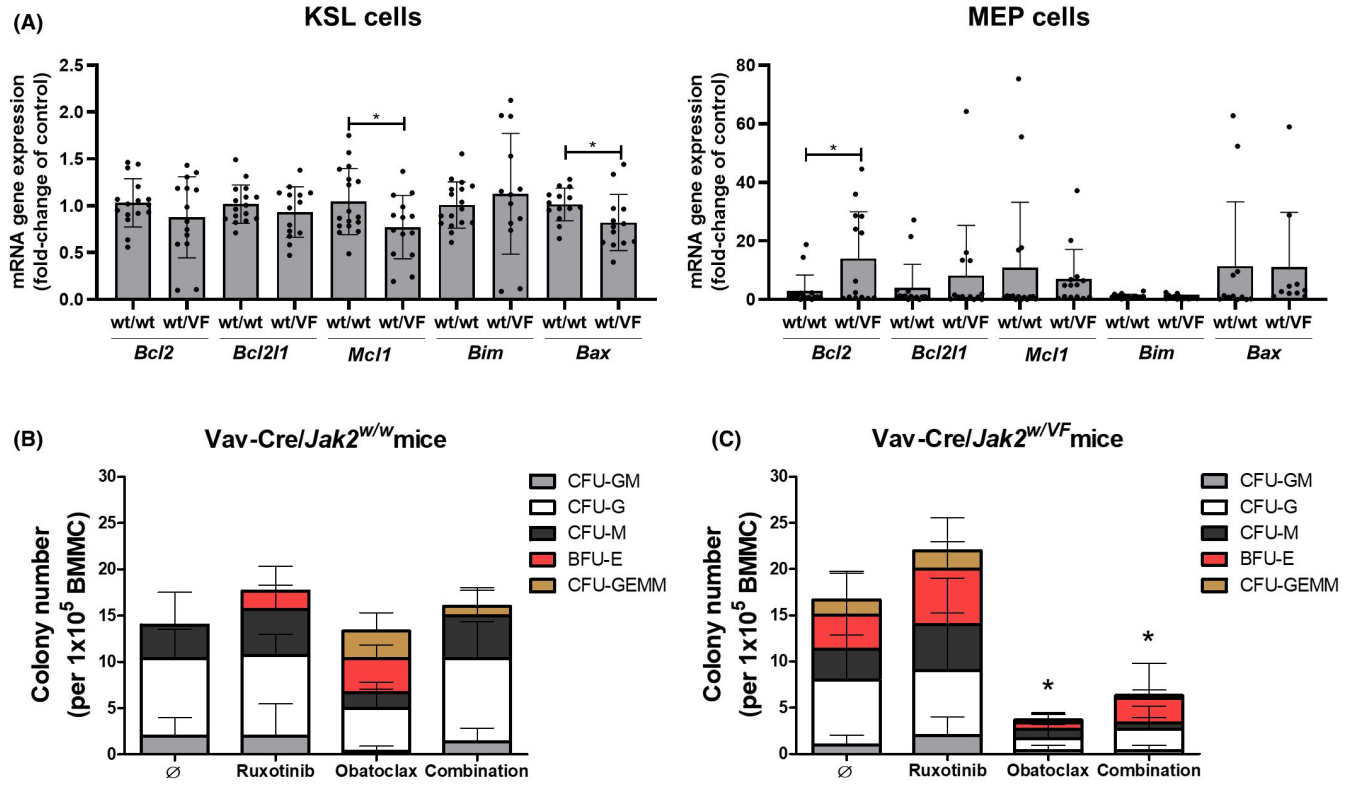


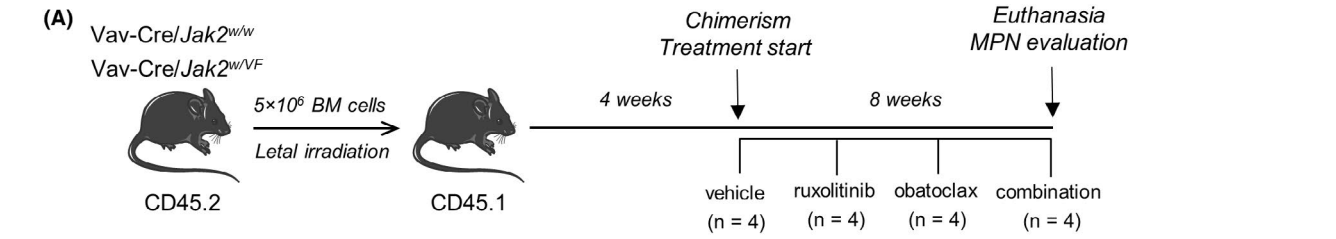
FIGURE 4 Differential expression of Bcl2 family genes in more immature and committed Jak2V617F hematopoietic MEP. A, Real-time PCR analysis of Bcl-2 family protein genes in KSL cells (left) and MEP cells (right) isolated from either *Vav-Cre/Jak2*$^{w/w}$ or *Vav-Cre/Jak2*$^{w/VF}$ mice. B, C, Colony-forming unit assays of BMMC isolated from *Vav-Cre/Jak2*$^{w/w}$ (B) and *Vav-Cre/Jak2*$^{w/VF}$ (C) mice in the presence or absence of ruxolitinib and/or obatoclax. Note that obatoclax efficiently lowers the frequencies of CFU-GM, CFU-G, CFU-M, BFU-E, and CFU-GEMM colonies from *Vav-Cre/Jak2*$^{w/VF}$ mice (C) compared with those from control mice (B). The bars represent mean ± SD values. Asterisks indicate *P*-value as follows: $^{*}P < .05$

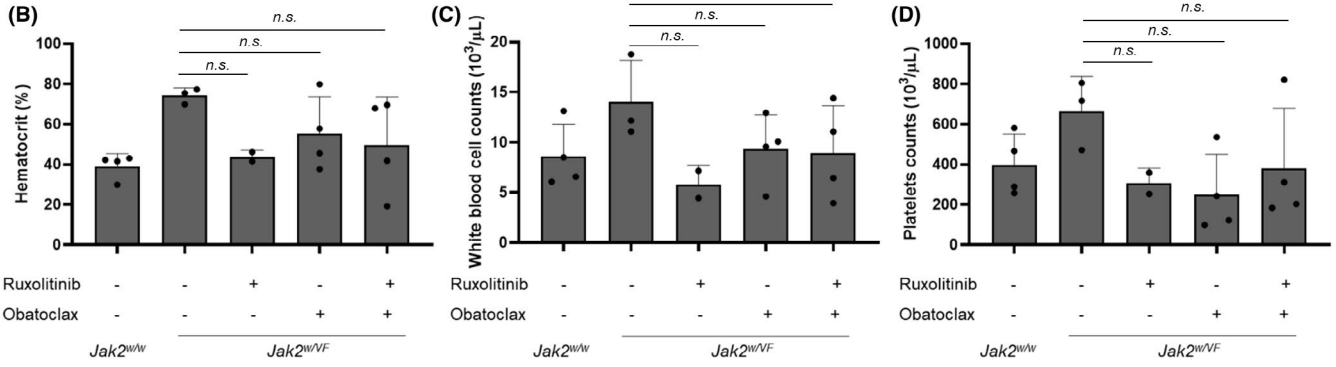
3.4 | Distinct survival mechanisms may be involved in Jak2V617F KSL and MEP populations

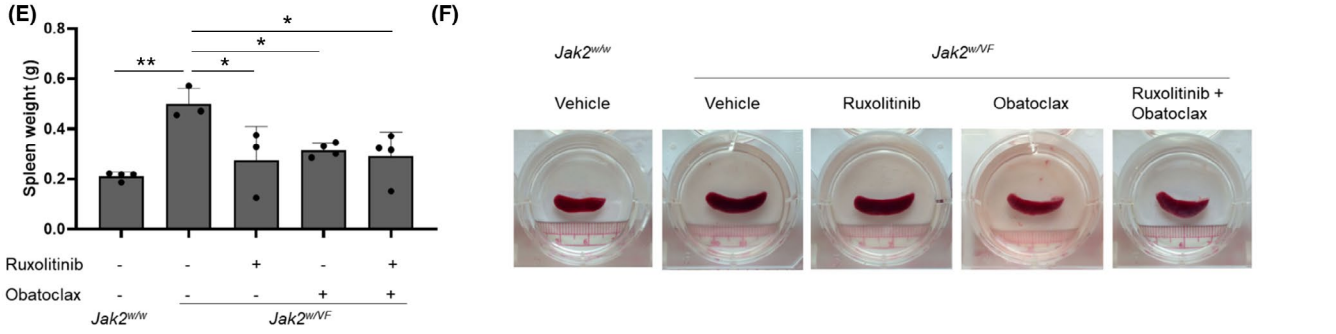
Previously, we have shown that Jak2 inhibition does not eradicate disease-initiating cells in the KSL population.[8] This observation prompted us to hypothesize that KSL (Lin^{-}Sca-1^{+}cKIT^{hi}) cells, progenitor cells, and more differentiated erythrocytes/megakaryocytes cells use distinct sets of BCL2 members. Therefore, we sorted KSL and MEP cells from either *Vav-Cre/Jak2*$^{w/w}$ or *Vav-Cre/Jak2*$^{w/VF}$ mice. Gene expression analysis revealed that expression of *Bcl2l1* in the KSL cells and MEP cells from *Vav-Cre/Jak2*$^{w/VF}$ mice was comparable with the ones from *Vav-Cre/Jak2*$^{w/w}$ mice (Figure 4A). Instead, expression of *Bax* was significantly downregulated in the KSL compartment (Figure 4A: left), whereas that of *Bcl2* was upregulated in MEP cells from *Vav-Cre/Jak2*$^{w/VF}$ mice (Figure 4A: right).

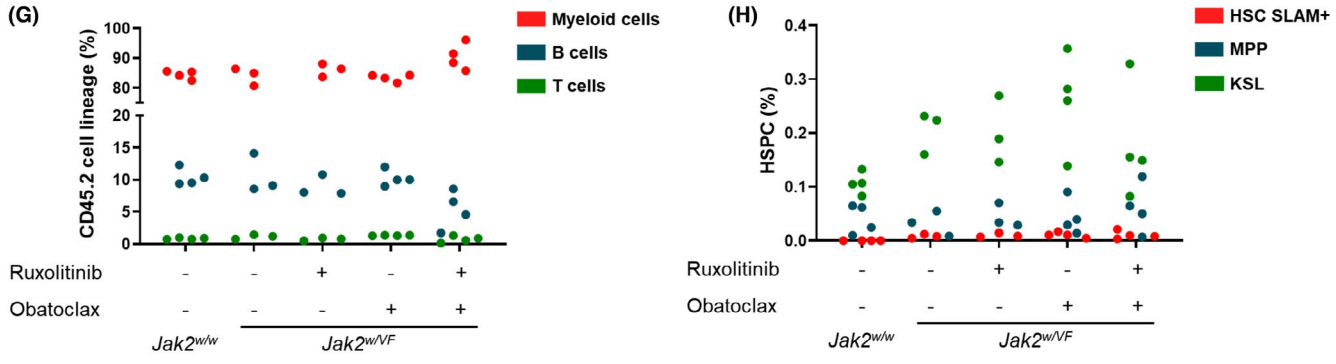
Next, after concluding that survival mechanisms in this model may be cell-type specific and depend on multiple BCL2 members, we investigated whether the pan-BCL2-family protein inhibitor obatoclax or its combination with ruxolitinib would reduce differential colony formation in *Vav-Cre/Jak2*$^{w/VF}$ compared with the controls. Ruxolitinib, used for comparison as a drug that directly target the JAK/STAT signaling, did not reduce the colony numbers, whereas BCL2 pan-inhibition with obatoclax was shown to efficiently reduces

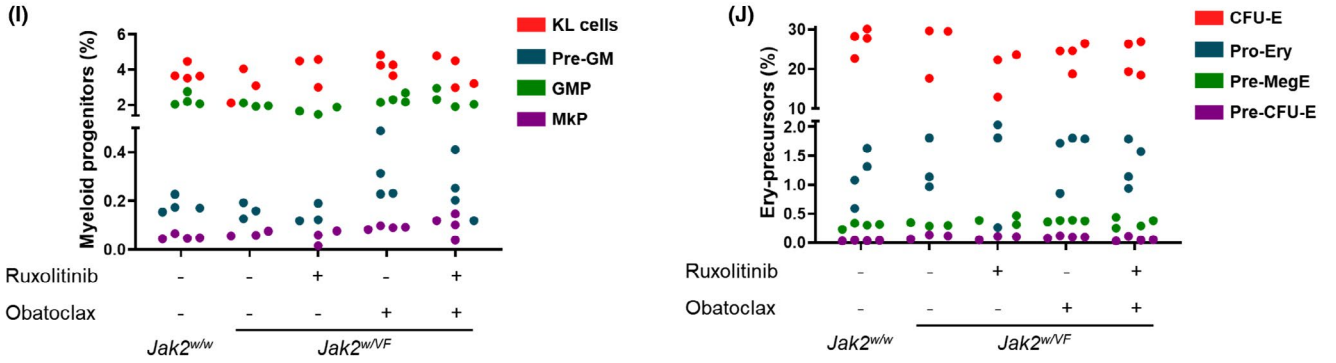
FIGURE 5 In vivo targeting of Bcl2 ameliorates disease burden but combined ruxolitinib and obatoclax treatment did not modulate the MPN phenotype in a Jak2V617F mouse model. A, Experimental design for induction of the Jak2V617F MPN phenotype in mice. Mice transplanted with Jak2$^{W/W}$ cells were used as the normal control. Bar graphs showing the (B) Ht levels, (C) WBC and (D) PLT counts of vehicle, ruxolitinib, obatoclax, or combined therapy-treated mice; ANOVA test and Bonferroni post-test. E, Spleen weight and (F) images of vehicle, ruxolitinib, obatoclax, or combined therapy-treated mice. B-E, Bars represent mean ± SD values. *n.s.*, not significant. Asterisks indicate *P*-value as follows: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. G, Flow cytometric enumeration of B220^{+} (B cells), CD3^{+} (T cells) and CD11b^{+}Gr1^{+} (myeloid cells) cells in the peripheral blood, (H) hematopoietic stem and progenitor cells, myeloid progenitors, and erythroid precursors of vehicle, ruxolitinib, obatoclax, or combined therapy-treated mice. CFU-E, colony-forming unit-erythroid; GMP, granulocyte-monocyte progenitor; HSC, hematopoietic stem cells; KL, c-Kit^{Hi}Sca-1^{-}Lin^{-}; KSL, c-Kit^{Hi}Sca-1^{+}Lin; MkP, megakaryocyte progenitor; MPP, multipotent progenitor; pre-CFU-E, colony-forming unit-erythroid precursor; pre-GM, granulocyte-monocyte precursor; pre-MegE, megakaryocyte-erythroid precursor; pro-Ery, pro-erythroblasts


(A)
Vav-Cre/Jak2w/w
Vav-Cre/Jak2w/VF
5×10^6 BM cells
Letal irradiation
CD45.2
CD45.1
Chimerism
Treatment start
Euthanasia
MPN evaluation
4 weeks
8 weeks
vehicle (n = 4)
ruxolitinib (n = 4)
obatoclax (n = 4)
combination (n = 4)


(B)
Hematocrit (%)
(C)
White blood cell counts (10^3/μL)
(D)
Platelets counts (10^3/μL)
n.s.
Ruxolitinib
Obatoclax
Jak2w/w
Jak2w/VF


(E)
Spleen weight (g)
(F)
Jak2w/w
Jak2w/VF
Vehicle
Vehicle
Ruxolitinib
Obatoclax
Ruxolitinib + Obatoclax


(G)
CD45.2 cell lineage (%)
Myeloid cells
B cells
T cells
(H)
HSPC (%)
HSC SLAM+
MPP
KSL
Ruxolitinib
Obatoclax
Jak2w/w
Jak2w/VF


(I)
Myeloid progenitors (%)
KL cells
Pre-GM
GMP
MkP
(J)
Ery-precursors (%)
CFU-E
Pro-Ery
Pre-MegE
Pre-CFU-E
Ruxolitinib
Obatoclax
Jak2w/w
Jak2w/VF

the frequencies of all colonies (CFU-GM, CFU-G, CFU-M, BFU-E, and CFU-GEMM; Figure 4C). Those effects were not observed in *Vav-Cre/Jak2*$^{w/w}$ (Figure 4B).

3.5 | Targeting Bcl2 by in vivo treatment of *Jak2*$^{w/VF}$ mice with the BH3 mimetic inhibitor obatoclax ameliorates the myeloproliferative disease phenotype

Next, we wanted to assess the efficacy of obatoclax or its combination with ruxolitinib in vivo, we generated a syngeneic transplantation model as outlined in Figure 5A. At 4 wk after BM transplantation, mice were randomized according to peripheral blood CD45.1/CD45.2 chimerism in the following treatment groups: vehicle, ruxolitinib, obatoclax, or drug combination. Treatment was administered to transplant recipients for 8 wk (Figure 5A). At 8 wk after treatment, either ruxolitinib or obatoclax alone, or a combination of both showed a trend, but failed to significantly reduce Hb/Ht, WBC and PLT (Figure 5B-D). However, we observed that obatoclax alone significantly reduced spleen weight as much as ruxolitinib (Figure 5E,F). There was no synergistic effect with the drug combination. The tumor burden reduction observed with either one of the drugs seemed to affect terminal differentiation stage neoplastic cells globally rather than myeloid-erythroid precursors, as shown by the immunophenotypic quantification of hematopoietic stem and progenitor cells after euthanasia (Figure 5G-J).

4 | DISCUSSION

JAK2V617F plays a central role in MPN initiation and maintenance by promoting cell proliferation and inhibiting apoptosis through activating its downstream signaling. In this regard, JAK2V617F is considered as a "driver oncogene" for MPN. However, a strategy to inhibit JAK2 activity has not reached a successful core for MPN per se, although it helps relieve splenomegaly and constitutional symptoms. Therefore, a better strategy to treat MPN is sorely needed.

In addition to development of more potent and effective JAK2 inhibitors, it is a reasonable approach to manipulate the major signaling pathways downstream of JAK2V617F. As BCL-XL is overexpressed in erythroid precursors cells from PV patients,[2] this anti-apoptotic BCL-2 family member has drawn attention to be one of the critical molecules for pathogenesis of MPNs.[3,4] Consistent with this notion, we and others have shown that BH3 mimetics in combination of JAK inhibitors or interferon α are effective in reducing erythroid colonies.[7,21] However, it was challenging to directly prove the pathological role of BCL-XL on MPN development in vivo, as several models of conditional deletion of *Bcl2l1* have shown an anemic phenotype with splenomegaly due to excessive apoptosis during late-stage erythropoiesis.[18-20] Our conditional *Bcl2l1* knockout in *Jak2*$^{w/VF}$ mouse models revealed that the addition of JAK2^{V617F} failed to prevent apoptosis during late-stage erythropoiesis as well as megakaryopoiesis (Figure 2). Bcl-xl does not seem to play a pivotal role in overproduction of early-stage erythrocytes or megakaryocytes due to the following observations: (a) no mitigation of erythrocythemia was observed in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/f}$ mice, which express 50% of *Bcl2l1* compared with that in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/w}$ mice (Figure 2A); (b) hypercellularity with these cells was still observed and no dramatic reduction of MEP cells was detected in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ mice (Figure 2B); and (c) splenomegaly was more severe in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ mice than in *Mx1-Cre/Jak2*$^{w/w}$*/Bcl2l1*$^{f/f}$ mice (Figure 2C). However, we can learn from these findings that: (a) Bcl-xl haploinsufficiency is not enough to reduce peripheral blood counts; (b) myeloid precursors were not affected by Bcl-xL modulation, therefore suggesting that its effects are more dependent on a late-stage control of hematopoiesis; (c) Bcl-xl complete deletion led to a very severe pro-apoptotic phenotype with compensatory hypersplenism (as previously described for the Bcl2l1 knockout murine model) and therefore Jak2V617F-induced myeloid proliferation can explain the additional splenomegaly observed in the double transgenic model. Although it was clear that Jak2V617F mutation was not able to revert the Bcl-xL deficiency anemic phenotype, our main question was could Bcl-xL abrogation modify the myeloid expansion of the Jak2V617F model. When *Bcl2l1* was conditionally and completely deleted (*Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$), Ht, WBC and PLT counts did reduce significantly compared to Mx1-Cre/Jak2$^{w/VF}$/Bcl2l1$^{w/w}$, again suggesting that a terminal pro-apoptotic effect may result from BCL2 modulation and that this can be an option for controlling the MPN phenotype (Figure 2A).

Interestingly, an increase in leukocytes seems to be dependent on murine models, as WBC counts were increased in *Mx1-Cre* mice but not *Vav-Cre* mice (Figure 1A vs. Figure 2A). The exact mechanism of this discrepancy remains unknown, but the WBC increase in *Mx1-Cre* mice is likely to be Bcl-xL dependent, as leukocytosis was mitigated in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/f}$ mice compared with *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{w/w}$ mice.

Given that a broader range of the anti-apoptotic and pro-apoptotic Bcl-2 family members may be involved in production of MPN cells (Figure 3A), we decided to use cell line models to examine whether inhibition of multiple anti-apoptotic BCL-2 family members was sufficient to induce growth arrest and cell death. Among the JAK2V617F-positive cellular models, HEL cells showed greater sensitivity to obatoclax when compared with SET2 cells. Notably, HEL cells showed resistance to ruxolitinib-induced apoptosis,[22] highlighting a potential application of BCL-2 inhibitors in this context. HEL cells have a greater degree of malignity, being derived from a patient with erythroleukemia,[23] while SET2 cells are highly sensitive to apoptosis induced by ruxolitinib and are derived from a patient with essential thrombocythemia after leukemic transformation.[24] Together, these findings suggested that inhibition of BCL-2 proteins can be an alternative approach to induce apoptosis in cells harboring JAK2V617F.

Long-term remission or curative therapies for MPN must reach the hematopoietic stem cell (HSC) pool, AS these cells are able to initiate and sustain the neoplastic phenotype.[25] Therefore, a better understanding of the molecular basis of resistance to apoptosis in

MPN HSC is of interest to pave the way for new therapeutic strategies. Inhibition of JAK2^{V617F} is not sufficient to eliminate MPN HSC or progenitor cells[8] and, in addition, our results indicated that genetic deletion of *Bcl2l1* failed to reduce MPN progenitor cells in *Mx1-Cre/Jak2*$^{w/VF}$*/Bcl2l1*$^{f/f}$ mice, which indicates a compensatory effect of other members of the BCL-2 family. In fact, it has already been shown that MCL1 is capable of sustaining cell viability in the presence of BCL2 inhibition in myeloid neoplasms.[26] We have also observed that there is differential expression of Bcl2 family genes in more immature and committed Jak2V617F hematopoietic MEP, suggesting that survival mechanisms in this model may be cell-type specific and depend on multiple BCL2 members. Indeed, our experiments of directly targeting BCL2 with obatoclax, a pan-BCL-2 inhibitor, revealed that it inhibited MPN phenotypes in vitro (Figure 4B,C) and in vivo (Figure 5A-F) as a single drug, regardless of ruxolitinib addition. In agreement with genetic deletion of *Bcl2l1* results, obatoclax treatment resulted in a partial control of the myeloid expansion by reducing blood counts and spleen size in Jak2V617F transplanted mice, although not affecting HSC and progenitor cells.

In summary, our results demonstrated that manipulating the intrinsic apoptotic pathway may be an alternative strategy to treat MPN induced by JAK2V617F. Disrupting the BCL2 balance is not sufficient to treat MPN at the stem cell level, but it is certainly an option for controlling the myeloid expansion that leads to clinical complications dependent on increased cell counts and splenomegaly. This may be relevant to intolerant or resistant Jak2 inhibitor patients. Further studies are still required to develop novel strategies able to cure MPN.

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DISCLOSURE

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SUPPORTING INFORMATION

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