A blockade of leukotriene-mediated Alox5 function provides a new strategy for the treatment of *JAK2*V617F-induced polycythemia vera

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Abstract

Polycythemia vera (PV) is a hematopoietic stem cell disorder characterized by *JAK2*V617F, a mutation that gives rise to erythrocytosis. Our previous studies have shown that arachidonate 5-lipoxygenase (Alox5) is a critical driver in PV and the inhibition of the Alox5 pathway attenuates *JAK2*V617F-induced PV development in mice. However, the molecular mechanism underlying the function of Alox5 in PV remains elusive. It is well established that leukotrienes are important downstream molecules synthesized through the Alox5 pathway in leukocytes and they play a key role in mediating Alox5 functions in human asthma. In this study, we found that Montelukast, a leukotriene receptor antagonist, inhibits cell proliferation and induces apoptosis in *JAK2*V617F-cell lines. Further *in vivo* studies demonstrated that Montelukast treatment suppresses the development of PV induced by *JAK2*V617F in mice, comparable to the effect of Alox5 inhibition on PV development. Notably, the inhibitory effect of Montelukast on PV is dependent on selectively eradicating PV stem cells while sparing their normal counterparts. Moreover, we found that Montelukast synergizes with the JAK2 inhibitor (ruxolitinib) to inhibit proliferation of *JAK2*V617F-expressing hematopoietic cells *in vitro* and in *JAK2*V617F mice. Finally, we showed that Montelukast induces caspase-3- and PARP-associated apoptosis of *JAK2*V617F-expressing cells. These findings indicate that Montelukast is a promising candidate agent and could be combined with an JAK2 inhibitor (such as Ruxolitinib) for PV treatment.

Introduction

Myeloproliferative neoplasms (MPN) comprise a group of hematologic malignancies that arise from hematopoietic stem cells (HSC).[1,2] Classical MPN include chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[3-6] PV, the most common MPN, is a clonal stem cell disease characterized by a plethoric appearance in clinic with an absolute erythrocytosis.[7] This disease can be complicated by leukocytosis, thrombocytosis, and a propensity for progression to myelofibrosis and acute myeloid leukemia.[8] In 2005, four different research groups identified an identical mutation in *JAK2* tyrosine kinase (*JAK2*V617F) in the majority of patients with PV and a significant proportion of patients with ET and PMF.[9-12] JAK2 is a member of the Janus family of non-receptor tyrosine kinases and is involved in the signaling of type I cytokine receptors such as the erythropoietin (EPO) receptor, thrombopoietin receptor (TPO), and granulocyte macrophage colony-stimulating factor (GM-CSF) receptor.[13] Under physiological conditions, JAK2 is activated after cytokines bind to receptors, which in turn phosphorylates the downstream signal transducer and activator of transcription (STAT), leading to activation of the JAK2-STAT pathway.[14] Protein structural analysis shows that the somatic V617F mutation disrupts the autoinhibitory state of JAK2, resulting in the constitutive activation of the JAK2 tyrosine kinase in the absence of cytokines.[10] This acquired somatic mutation results in constitutive activation of downstream signaling pathways including the JAK2-STAT signaling and transforms growth factor-dependent hematopoietic cells to cytokine-independence.[15-18]

Arachidonate 5-lipoxygenase (Alox5) has been reported

to regulate numerous physiological and pathological progresses, including inflammation and cancer.[19,20] Our previous study showed Alox5 as a key effector of *JAK2*V617F in driving PV. Alox5 is upregulated by *JAK2*V617F and genetic deletion of Alox5 moderates PV development induced by *JAK2*V617F in mice.[21] Additionally, Alox5 inhibition reduces the clonal growth of human *JAK2*V617F-expressing $CD34^+$ cells.[21] These results suggest Alox5 as a candidate therapeutic target for *JAK2*V617F-induced PV therapy. However, the molecular mechanism by which Alox5 inhibition attenuates *JAK2*V617F-induced PV remains unclear. Montelukast, a Food and Drug Adminstration-approved anti-human asthma drug, functions as a type 1 cysteinyl leukotriene receptor (CysLTR1) antagonist that blocks the action of leukotrienes to reduce inflammation response.[22] Leukotrienes are a family of eicosanoid inflammatory mediators synthesized from arachidonic acid (AA) by 5-lipoxygenase (5-LO) encoded by Alox5.[23-25] Leukotrienes serve as important downstream lipid metabolites within the Alox5 pathway and are involved in inflammation, allergy, as well as in metabolism of some cancer cells.[26-28] Although our previous data showed Alox5 as a potential target for suppressing PV development, it is unclear what roles leukotrienes play in Alox5-mediated PV pathogenesis. Here, we investigated the efficacy of Montelukast against PV using a *Jak2*V617F knock-in mouse model.[15] We demonstrated the effect of leukotrienes on *JAK2*V617F-expressing PV cells using the CysLTR1 antagonist Montelukast both *in vitro* and *in vivo*, proposing Montelukast as a promising therapeutic agent for treatment of PV.

Methods

Cell lines and mice

A *JAK2*V617F-GFP-expressing murine BaF/3 cell line was kindly provided by Dr. Ross Levine (Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY). The human *JAK2*V617F-expressing erythroleukemia (HEL) cell line was purchased from ATCC. All cell lines were cultured in RPMI-1640 medium containing 10% fetal calf serum and 50 μmol/L 2-mercaptoethanol. BaF/3 cells were cultured in the same medium with the addition of 1:100 murine recombinant interleukin (IL)-3. All animal work was approved by our Institutional Animal Care and Use Committee.

Retroviral bone marrow transplantation model of polycythemia vera

The pMSCV-*JAK2*V617F-GFP construct was kindly provided by Dr. Richard Van Etten (Tufts-New England Medical Center). The *JAK2*V617F retrovirus was produced by co-transfection of HEK293T cells with pMSCV-*JAK2*V617F-GFP and ecotropic packaging construct. PV was induced in mice as follows: bone marrow (BM) cells from 5-FU–treated (200 mg/kg) donor mice were transduced twice with *JAK2*V617F retrovirus in the presence of IL-3, IL-6, and stem cell factor (SCF). The recipient mice received 1,100 cGy lethal γ irradiation (given by 2 split 550-cGy doses) and were then transplanted with $2.0x10^6$ cells via tail vein injection.

Flow cytometric analysis of hematopoietic cells

Hematopoietic cells were collected from peripheral blood and BM of PV mice, and red blood cells (RBC) were eliminated using RBC lysis buffer (pH 7.4). Then the cells were stained with fluorophore-labeled antibodies and subjected to flow cytometric analysis. All flow cytometry antibodies were purchased from eBiosciences.

Drug treatment

PV mice with disease confirmed by flow cytometry data or *JAK2*V617F transgenic mice were randomly placed into either placebo or drug treatment groups 5 months after BM transplantation (BMT). Montelukast (Camber Pharmaceuticals) was dissolved in water and given orally in a volume of 0.3 mL by gavage (150 mg/kg once daily) starting from 1 month after BMT. Ruxolitinib was administrated orally at 90 mg/kg, and water was used as placebo.

Cell cycle and apoptosis assays

Cells were plated in 12-well plates and treated with various doses (30-60 μm) of montelukast for 24 hours (h) and 48 h. Cells were stained with either propidium iodide (PI) for the cell cycle assay, or Annexin V and 7-AAD for the apoptosis assay. All results are expressed as the mean ± standard deviation.

Stem cell culture

Total BM cells isolated from PV mice were plated at a density of 10^6 cells/mL in StemSpan SFEM medium (STEMCELL™ Technologies #09650) supplemented with 10 ng/mL mouse SCF, 20 ng/mL mouse TPO, 10 ng/mL mouse FGF-1, 20 ng/mL mouse IGF-2, and 10 μg/mL heparin.

Western blot analysis

Unconjugated primary antibodies against AKT, JAK2, Caspase-3, and PARP were purchased from Cell Signaling Technology. A peroxidase-conjugated secondary antibody, in combination with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo-Fisher), was used to visualize phospho-JAK2. DyLight 800-conjugated and SB700-conjugated (Bio-Rad) secondary antibodies were used to visualize all other targets except β-actin, for which a rhodamine-conjugated primary antibody was used.

Statistical analysis

Student's *t* test was used for statistical analyses (GraphPad Prism; $*P<0.05$; $**P<0.01$; $***P<0.001$).

Results

Montelukast inhibits growth and induces apoptosis of *JAK2*V617F-expressing cells

We first assessed the effect of Montelukast on *JAK2*V617F-expressing cell lines *in vitro* using *JAK2*V617F-expressing mouse BaF/3 cells and human HEL cells. The cells were treated with various doses of Montelukast for 24 or 48 h, and we observed dose-dependent growth inhibition at both time points (Figure 1A). To explain how Montelukast inhibited cell proliferation, we performed cell cycle analysis and apoptosis assay using flow cytometric analysis. We found that Montelukast treatment blocked cell-cycle progression from G1 to S phase and from S to G2-M phase in both *JAK2*V617F-expressing BaF/3 cells and HEL cells (Figure 1B). Montelukast treatment also induced apoptosis of these cells (Figure 1C). Together, these results indicate that Montelukast inhibits cell growth through inhibition of cell-cycle progression and induces apoptosis of *JAK2*V617F-expressing cells, providing supportive evidence that Montelukast is a potential therapeutic agent for PV.

Montelukast suppresses polycythemia vera development in mice

To demonstrate an *in vivo* effect of Montelukast against PV, we induced PV in C57BL/6 mice by transplanting BM cells transduced with *JAK2*V617F retrovirus (pMSCV-*JAK2*V617F-GFP) into lethally-irradiated syngeneic recipient mice[21] and then treated the PV mice with a placebo or Montelukast for 4 months, during which the treated mice were monitored for PV development by flow cytometry and cell counts in peripheral blood. We observed that the percentage and number of $GFP^+Gr\text{-}1^+$ PV cells from Montelukast-treated mice increased much slowly than those from placebo-treated PV mice, beginning 2 months after the treatment (Figure 2A, top panel). In contrast, the percentage and number of normal $Gr\text{-}1^+$ cells ($GFP^-Gr\text{-}1^+$) between placebo- and Montelukast-treated PV mice were similar (Figure 2A, bottom panel), suggesting that Montelukast specifically suppressed the PV cells with no inhibitory effect on normal myeloid cells. The inhibitory effect of Montelukast on $GFP^+Gr\text{-}1^+$ PV cells was further confirmed by normalizing to the levels of cell counts for RBC and platelets in peripheral blood of the treated PV mice (*Online Supplementary Figure S1*). The inhibitory effect of Montelukast on PV mice was also supported by the reduced spleen weight (Figure 2B), likely due to less PV cell infiltration to the spleens. Because BM fibrosis is a devastating medical consequence of PV in patients and *JAK2*V617F induces fibrosis in BM of PV mice,[29] we compared the formation of BM fibrosis between Montelukast- and placebo-treated PV mice. We observed that the severity of fibrosis in Montelukast-treated PV mice was much less than that in placebo-treated mice (Figure 2C), suggesting that Montelukast treatment inhibits the progression of PV.

Montelukast inhibits polycythemia vera-initiating cells in mice

Our previous study in mice showed that Alox5 is required for the functions of leukemia stem cells in chronic myeloid leukemia,[30] a type of human myeloid proliferative neoplasm with the pathological features similar to PV. Therefore, we hypothesized that targeting of the Alox5 pathway by a blockade of the produced leukotrienes with Montelukast impedes the functions of PV-initiating cells. To test this hypothesis, BM cells were collected from PV mice after 4 months of treatment and analyzed by flow cytometry. We observed dramatic decreases in the percentage and number of GFP^+ PV cells in BM of Montelukast-treated but not placebo-treated mice (Figure 3A, top panel). In these mice, Montelukast did not show an inhibitory effect on the percentage and number of normal (GFP^-) BM cells (Figure 3A, bottom panel). Next, we analyzed PV-initiating cells (GFP^+LSK: $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$) in BM of Montelukast-treated PV mice in comparison with placebo-treated PV mice and observed that Montelukast caused a significant reduction of the percentage and number of BM GFP^+LSK cells in PV mice (Figure 3B, top panel). Montelukast did not inhibit normal (GFP-) BM LSK cells in PV mice (Figure 3B, bottom panel). These results suggest that Montelukast inhibits PV development through selectively inhibiting PV-initiating cells in mice. To further demonstrate the inhibitory effect of Montelukast on PV-initiating cells, we conducted a BMT experiment. We treated equal number of PV BM cells from *JAK2*V617F mice (CD45.2) *in vitro* with a placebo and Montelukast, respectively, under the stem cell conditions for 5 days. The BM cells from each treatment group were then divided equally and transplanted into three lethally-irradiated recipient mice along with co-transplantation of normal BM cells (CD45.1) into each recipient mouse. The goal of this approach is to assess more stringently the inhibitory effect of Montelukast on PV stem cell function by comparing the ability of the treated *JAK2*V617F BM cells to compete with normal BM cells in the same recipient mouse for chimerically repopulating the recipient mice, which will reflect the function of PV stem cells between the two treatment groups. Because stem cells functionally produce more mature cells in the periphery, we compared the chimeric ratios of CD45.2 cells (representing placebo- or Montelukast-treated *JAK2*V617F BM cells) and CD45.1 cells (representing normal BM cells) in peripheral blood of the recipient mice between the two treatment groups at 2, 3 and 4 weeks after the transplantation. We found that the chimeric ratios of CD45.2 cells and CD45.1 cells (CD45.2/CD45.1) in peripheral blood of Montelukast-treated group were significantly smaller than those in the placebo-treated group (Figure 3C, left two panels), indicating that the Montelukast treatment reduced the function of PV stem cells. At the 4-week time point, we sacrificed the mice to also analyze the BM cells from the two treatment groups of mice and found that the chimeric ratios of CD45.2 Gr-

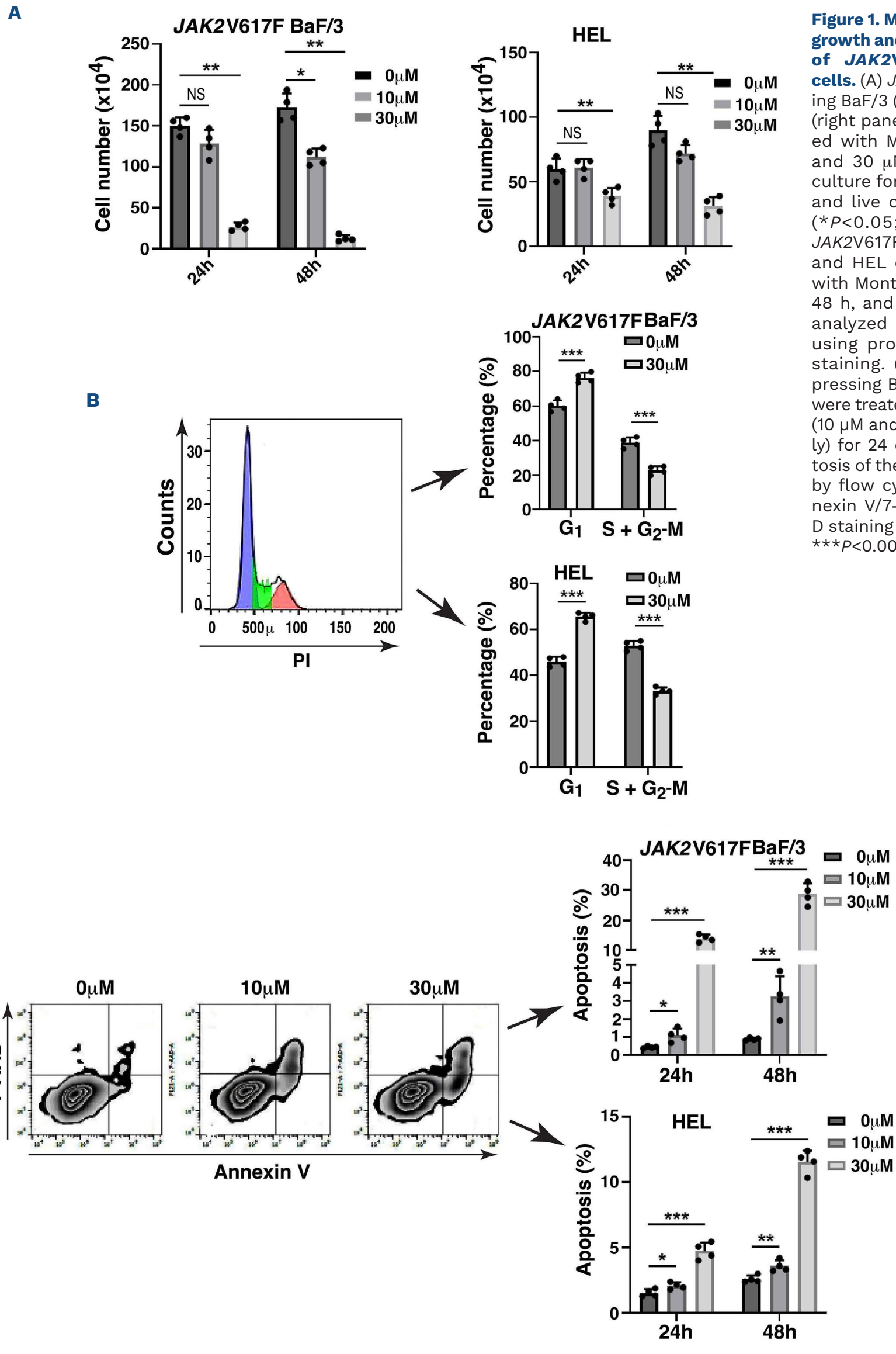


Figure 1. Montelukast inhibits growth and induces apoptosis of *JAK2*V617F-expressing cells. (A) *JAK2*V617F-expressing BaF/3 (left panel) and HEL (right panel) cells were treated with Montelukast (10 μM and 30 μM, respectively) in culture for 24 or 48 hours (h), and live cells were counted (**P*<0.05; ***P*<0.01). (B) *JAK2*V617F-expressing BaF/3 and HEL cells were treated with Montelukast (30 μM) for 48 h, and the cell cycle was analyzed by flow cytometry using propidium iodide (PI) staining. (C) *JAK2*V617F-expressing BaF/3 and HEL cells were treated with Montelukast (10 μM and 30 μM, respectively) for 24 or 48 h, and apoptosis of the cells was analyzed by flow cytometry using Annexin V/7-aminoactinomycin D staining (**P*<0.05; ***P*<0.01; ****P*<0.001).).

1+, LSK and LT-HSC cells were significantly smaller than those of CD45.1 Gr-1+, LSK and LT-HSC cells, supporting the inhibitory effect of Montelukast treatment on PV stem cells (Figure 3C, right three panels).

Because Montelukast is an antagonist of leukotriene receptor whose activation upon binding to leukotrienes mediate Alox5 functions, it is plausible to think that the inhibitory effect of Montelukast on PV-initiating cells is induced through blocking leukotriene binding to their receptors. To test this idea, we added leukotriene C4 (LTC4) and leukotriene D4 (LTD4) to Montelukast-treated culture of PV-initiating cells to see if leukotrienes could rescue cell proliferation inhibition caused by Montelukast. Total BM cells isolated from PV mice were treated with Mon-

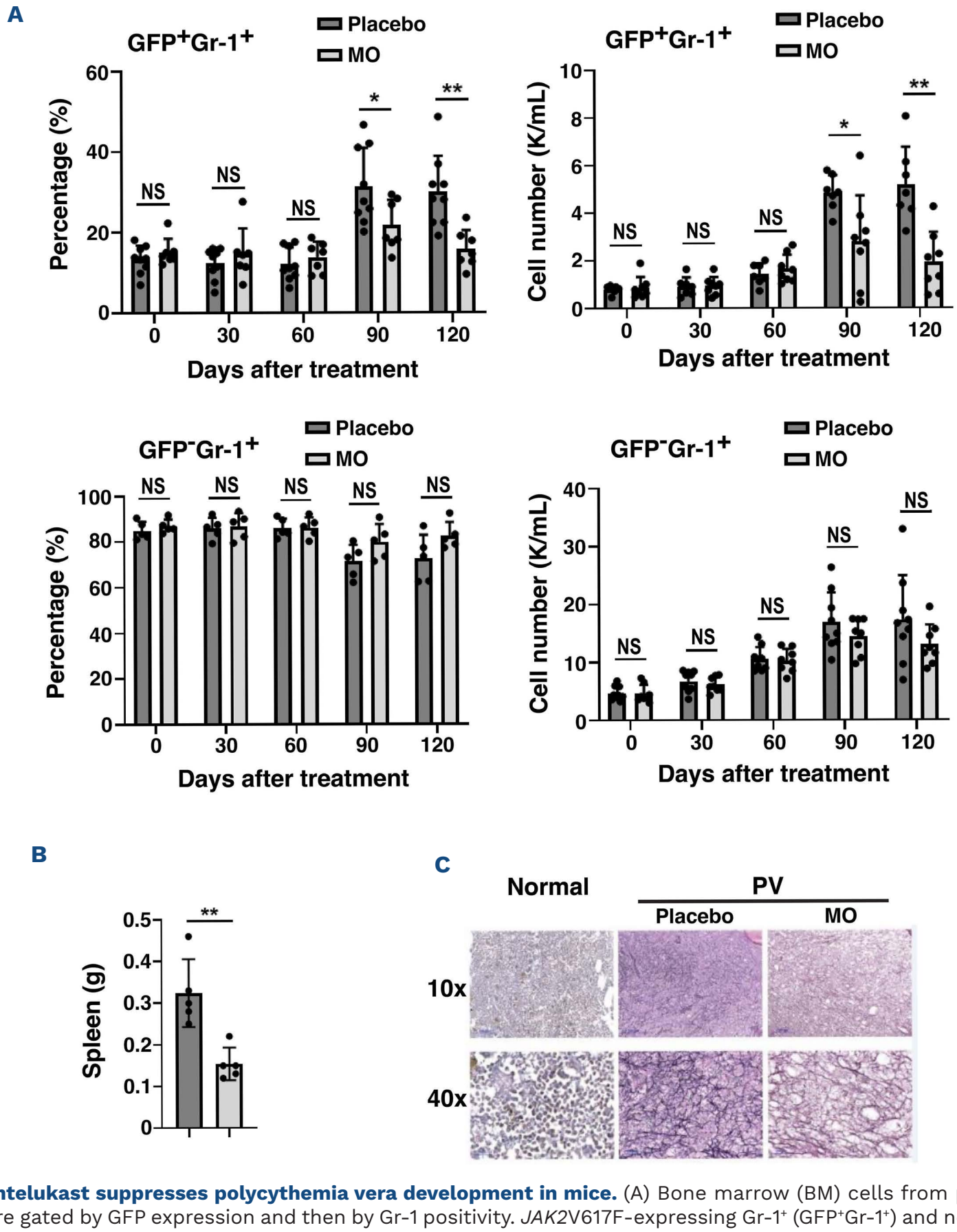
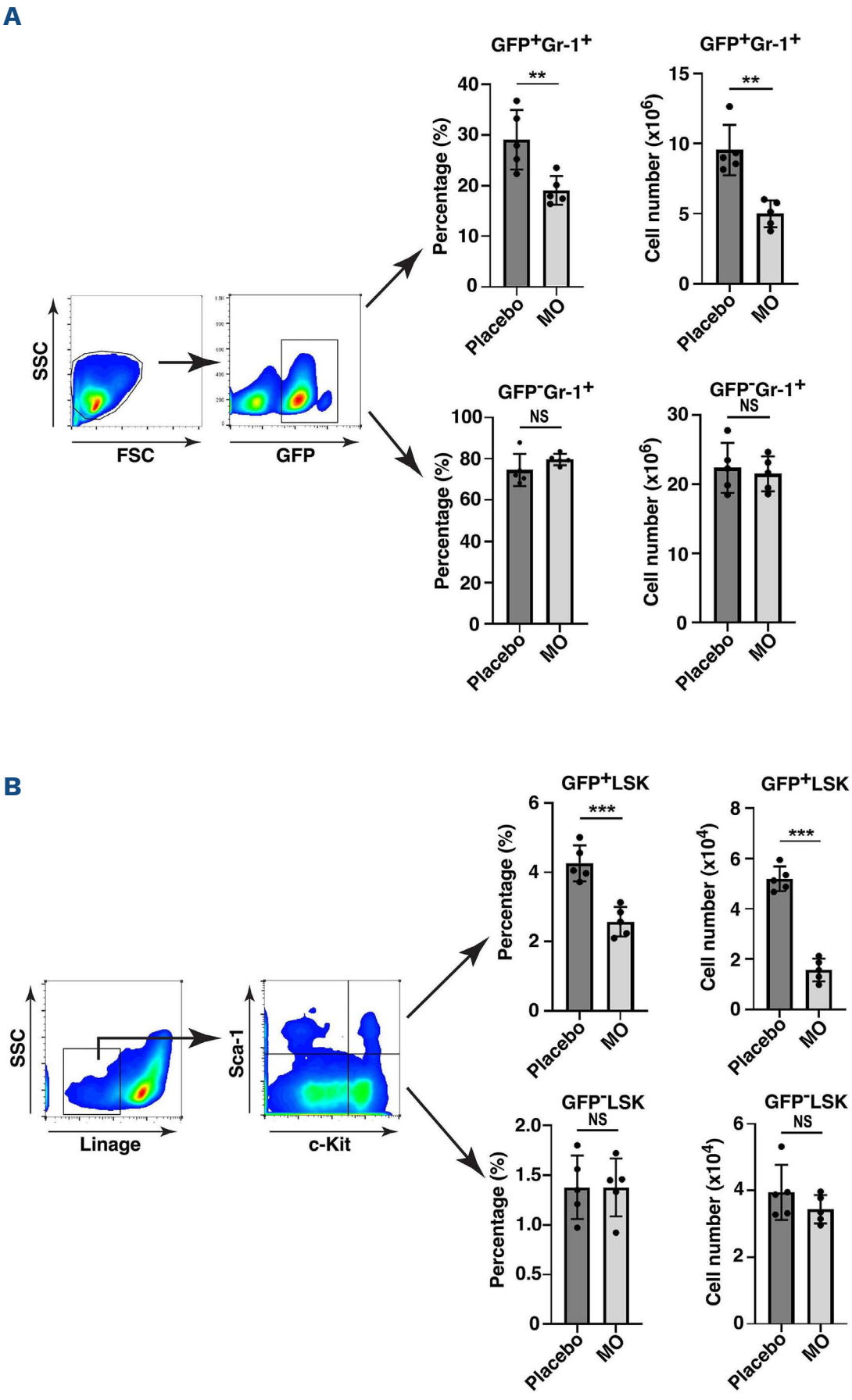


Figure 2. Montelukast suppresses polycythemia vera development in mice. (A) Bone marrow (BM) cells from polycythemia vera (PV) mice were gated by GFP expression and then by Gr-1 positivity. *JAK2*V617F-expressing Gr-1+ (GFP+Gr-1+) and non-*JAK2*V617F-expressing Gr-1+ (GFP-Gr-1+) cells were analyzed by flow cytometry and cell count for the percentage and number in peripheral blood of PV mice after treatment with a placebo or Montelukast (MO) at different time points. A significant decrease in GFP+Gr-1+ but not GFP-Gr-1+ cells were observed with time (*P<0.05; **P<0.01; N=5 for each group). (B) Spleen weight of the PV mice treated with a placebo or Montelukast was compared (P<0.01; N=5). (C) BM tissue sections with silver staining were compared between placebo- and Montelukast-treated PV mice. Note that 10x and 40x are objective lenses. NS: not significant.



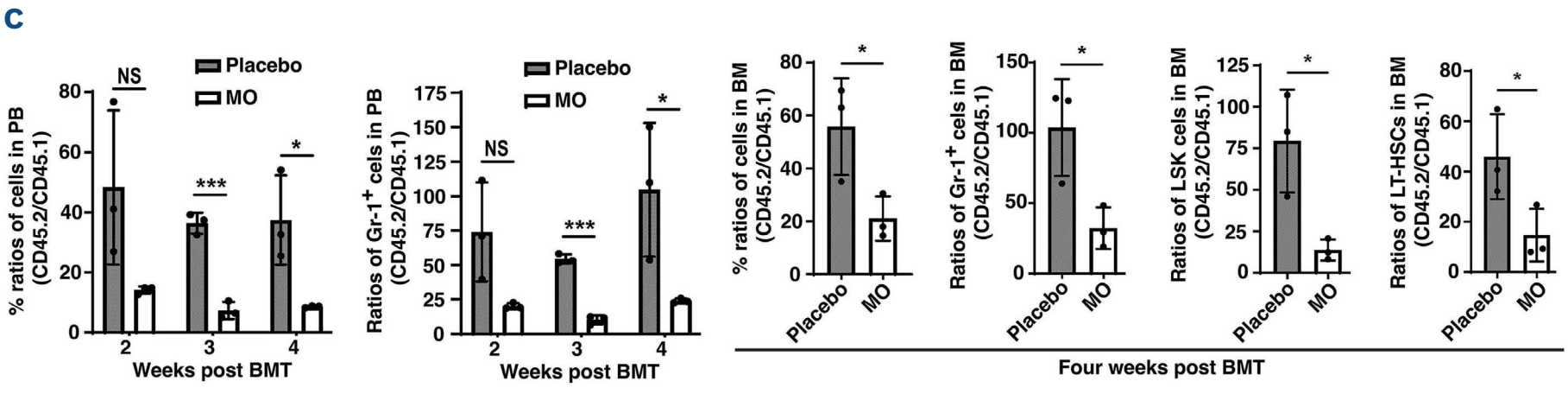


Figure 3. Montelukast inhibits polycythemia vera-initiating cells in mice. (A) Flow cytometry analysis showed significant decreases in the percentage (left panel) and number (right panel) of *JAK2*V617F-expressing Gr-1$^+$ (GFP$^+$Gr-1$^+$) but not non-*JAK2*V617F-expressing Gr-1$^+$ (GFP$^-$Gr-1$^+$) cells in the bone marrow (BM) of polycythemia vera (PV) mice treated with Montelukast (MO) (*P<0.05; **P<0.01; N=5 for each group). (B) Flow cytometry analysis showed significant decreases in the percentage (left panel) and number (right panel) of PV-initiating cells (LSK, GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) but not normal LSK cells (GFP$^-$Lin$^-$c-Kit$^+$Sca-1$^+$) in BM of PV mice treated with Montelukast (**P<0.01; ***P<0.001; N=5 for each group). (C) Equal number of PV BM cells from *JAK2*V617F mice *in vitro* with a placebo and Montelukast, respectively, under the stem cell conditions for 5 days. The BM cells (CD45.2) from each treatment group were then divided equally and transplanted into 3 lethally-irradiated recipient mice along with co-transplantation of normal BM marrow cells (CD45.1) into each recipient mouse (1x10^5 per recipient mouse). The chimeric ratios of CD45.2 cells (representing treated *JAK2*V617F BM cells) and CD45.1 cells (representing normal BM cells) in peripheral blood of recipient mice between the 2 treatment groups at 2, 3 and 4 weeks after the transplantation. The BM cells from the 2 treatment groups of mice were analyzed at 4 weeks after transplantation. BMT: bone marrow transplantation; NS: not significant.

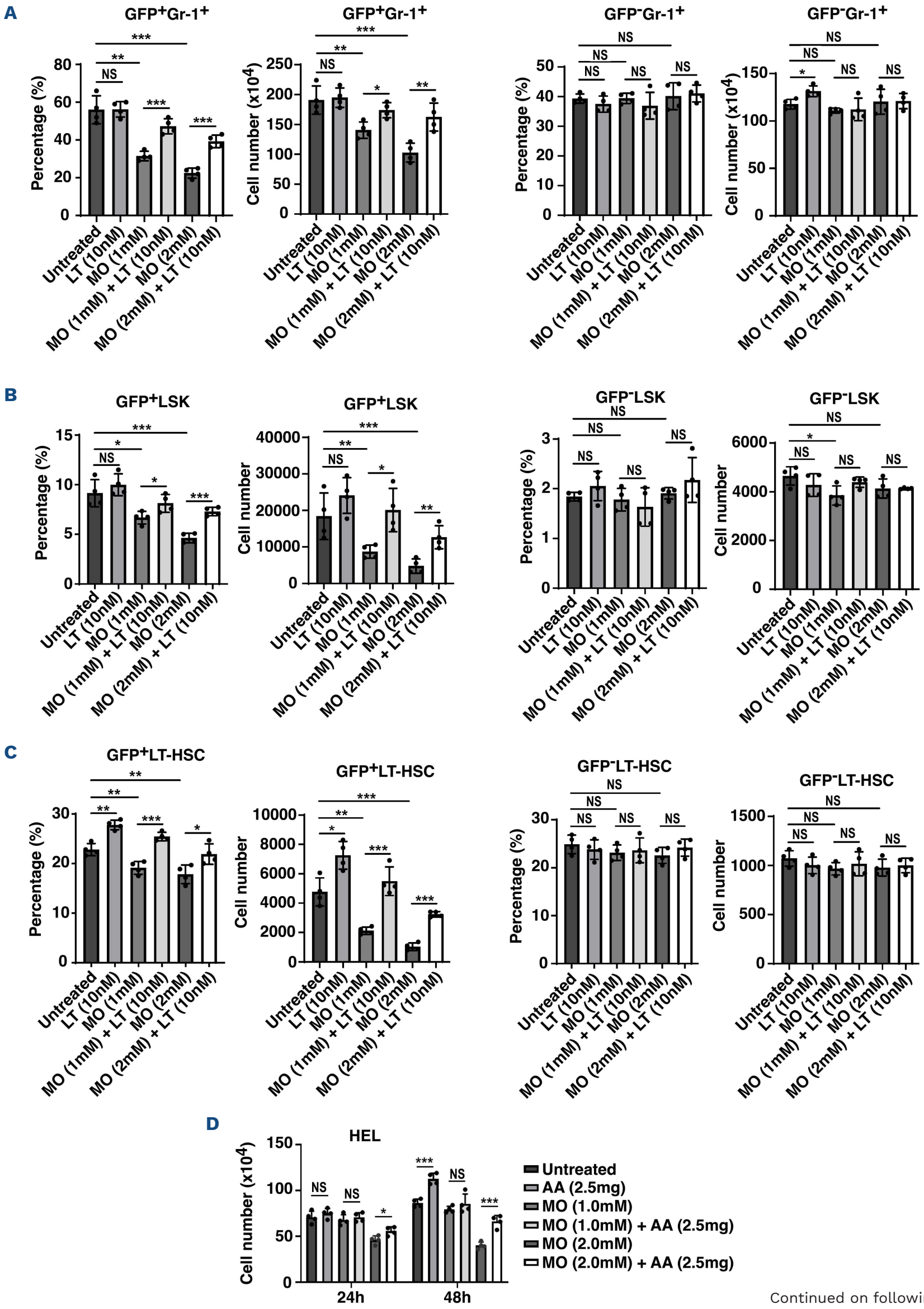


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Figure 4. Leukotrienes or arachidonic acid partially rescues the inhibitory effect of Montelukast on polycythemia vera cells. Bone marrow (BM) cells isolated from polycythemia vera (PV) mice were plated at a density of 10^6/mL in StemSpan SFEM media. The medium was replaced after 3 days in the presence of leukotrienes (LTC4 and LTD4) alone, Montelukast (MO) alone, or both for additional 2 days. (A) The percentage and number of *JAK2*V617F-expressing Gr-1$^+$ (GFP$^+$Gr-1$^+$) cells (left panel) were analyzed by flow cytometry and cell count (*P<0.05; **P<0.01; N=3 for each group) compared to those of non-*JAK2*V617F-expressing Gr-1$^+$ (GFP-Gr-1$^+$) cells (right panel). (B) BM cells isolated from PV mice were plated at a density of 10^6/mL in StemSpan SFEM media. The medium was replaced after 3 days in the presence of leukotrienes alone, Montelukast alone, or both for additional 2 days. The percentage and number of *JAK2*V617F-expressing LSK (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) cells (left panel) were analyzed by flow cytometry and cell count (*P<0.05; **P<0.01; N=3 for each group) compared to those of non-*JAK2*V617F-expressing LSK cells (GFP$^-$Lin$^-$c-Kit$^+$Sca-1$^+$) cells (right panel). (C) BM cells isolated from PV mice were plated at a density of 10^6/mL in StemSpan SFEM media. The medium was replaced after 3 days in the presence of LT alone, Montelukast alone, or both for additional 2 days. The percentage and number of *JAK2*V617F-expressing long-term hematopoietic stem cells (HSC) (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$) (left panel) were analyzed by flow cytometry and cell count (*P<0.05; **P<0.01; N=3 for each group) compared to those of non-*JAK2*V617F-expressing LT-HSC cells (right panel). (D) Human *JAK2*V617F-expressing HEL cells were plated at a density of 5x10^5/mL in serum-free media in the presence of Montelukast alone, Arachidonic acid (AA) alone, or both for 24 and 48 hours, respectively, and live cells were counted (*P<0.05; **P<0.01). NS: not significant.

telukast in the presence of both LTC4 and LTD4 for 2 days, respectively. We found that the percentages and numbers of *JAK2*V67F-expressing myeloid cells (GFP$^+$Gr-1$^+$) (Figure 4A, left panel), LSK cells (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) (Figure 4B, left panel), and long-term hematopoietic stem cells (HSC) (LT-HSC) (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$) (Figure 4C, left panel) decreases markedly with Montelukast treatment in comparison to untreated cells. This inhibitory effect, especially on PV-initiating cells (LSK and LT-HSC), was partially rescued by adding leukotrienes to the culture (Figure 4A-C, left panel). In contract, Montelukast did not have inhibitory effects on non-*JAK2*V617F-expressing (GFP$^-$) myeloid cells (GFP$^-$Gr-1$^+$) (Figure 4A, right panel), LSK cells (GFP$^-$Lin$^-$c-Kit$^+$Sca-1$^+$) (Figure 4B, right panel), and long-term hematopoietic stem cells (LT-HSC) (GFP$^-$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$) (Figure 4C, right panel). These results suggest that the inhibition of *JAK2*V617F mutant PV cells and PV-initiating cells in BM by Montelukast is largely due to a blockade of leukotriene binding to their receptors. To examine whether the blockade of leukotriene receptor binding by Montelukast is also a cellular mechanism for inhibiting human *JAK2*V617F-expressing cells, we treated human HEL cells with Montelukast in the presence and absence of arachidonic acid (AA) that gives rise to leukotrienes after being metabolized in the cells. We observed that the growth inhibition of HEL cells by Montelukast was largely rescued by the addition of AA to culture for 48 h (Figure 4D).

Montelukast synergizes with Ruxolitinib in suppression of polycythemia vera cells

Ruxolitinib is a JAK1/2 inhibitor that has been used to treat PMF and advanced PV patients through blocking the JAK-STAT pathway. Our results suggested that Montelukast suppresses *JAK2*V617F-expressing cells and PV development through inhibiting Alox5 function by blocking leukotriene binding to their receptors (Figures 1-4). Therefore, we thought that Montelukast would synergize with Ruxolitinib to suppress PV cells. To test this idea, we first treated HEL cells with Montelukast, Ruxolitinib, or both in culture and found that Montelukast acted synergistically with Ruxolitinib to inhibit cell growth (Figure 5A), block cell-cycle progression (Figure 5B) and induce cellular apoptosis (Figure 5C). Next, we tested the combined effects of Montelukast and Ruxolitinib *in vivo*. We treated *JAK2*V617F-expressing PV mice with a placebo, Montelukast, Ruxolitinib, and both Montelukast and Ruxolitinib, and 5 months after the treatments, we analyzed BM cells in the treated PV mice. Total number of BM cells was significantly reduced by the combined treatment with Montelukast and Ruxolitinib (Figure 5D). Furthermore, the percentage and number of PV-initiating LSK and LT-HSC cells were greatly reduced upon the combined treatment with Montelukast and Ruxolitinib (Figure 5E, F). To further demonstrate the synergistic inhibition of PV cells by the combined treatment with Montelukast and Ruxolitinib, we analyzed these results with the CompuSyn software (freely available online via Google) and confirmed the synergistic inhibitory effects on *JAK2*V617F-expressing HEL cells and PV cells in mice (*Online Supplementary Figure S2*). Taken together, these results indicate that Montelukast and Ruxolitinib have a synergistic effect on suppressing *JAK2*V617F-expressing cells and PV development.

On the other hand, we believe it will be significant to examine potential toxic effect of the combined treatment with Montelukast and Ruxolitinib, if we plan to translate our findings into future clinical use. Therefore, we treated wild-type mice with a placebo, Montelukast alone, Ruxoliyinib alone or Montelukast and Ruxolitinib for 1 month. We did not observe any toxic effects on mice based on our evaluation of the mice with body weight and blood cell counts (*Online Supplementary Figure S3*).

Caspase-3 and PARP are involved in Montelukast-induced apoptosis of *JAK2*V617F-expressing cells

Because Montelukast induced apoptosis of *JAK2*V617F-expressing cells (Figure 1C), we wondered which apoptotic pathway is involved. We treated HEL cells with Montelukast in culture and found that protein degradation of Caspase-3 and PARP was enhanced, as shown by increased levels of

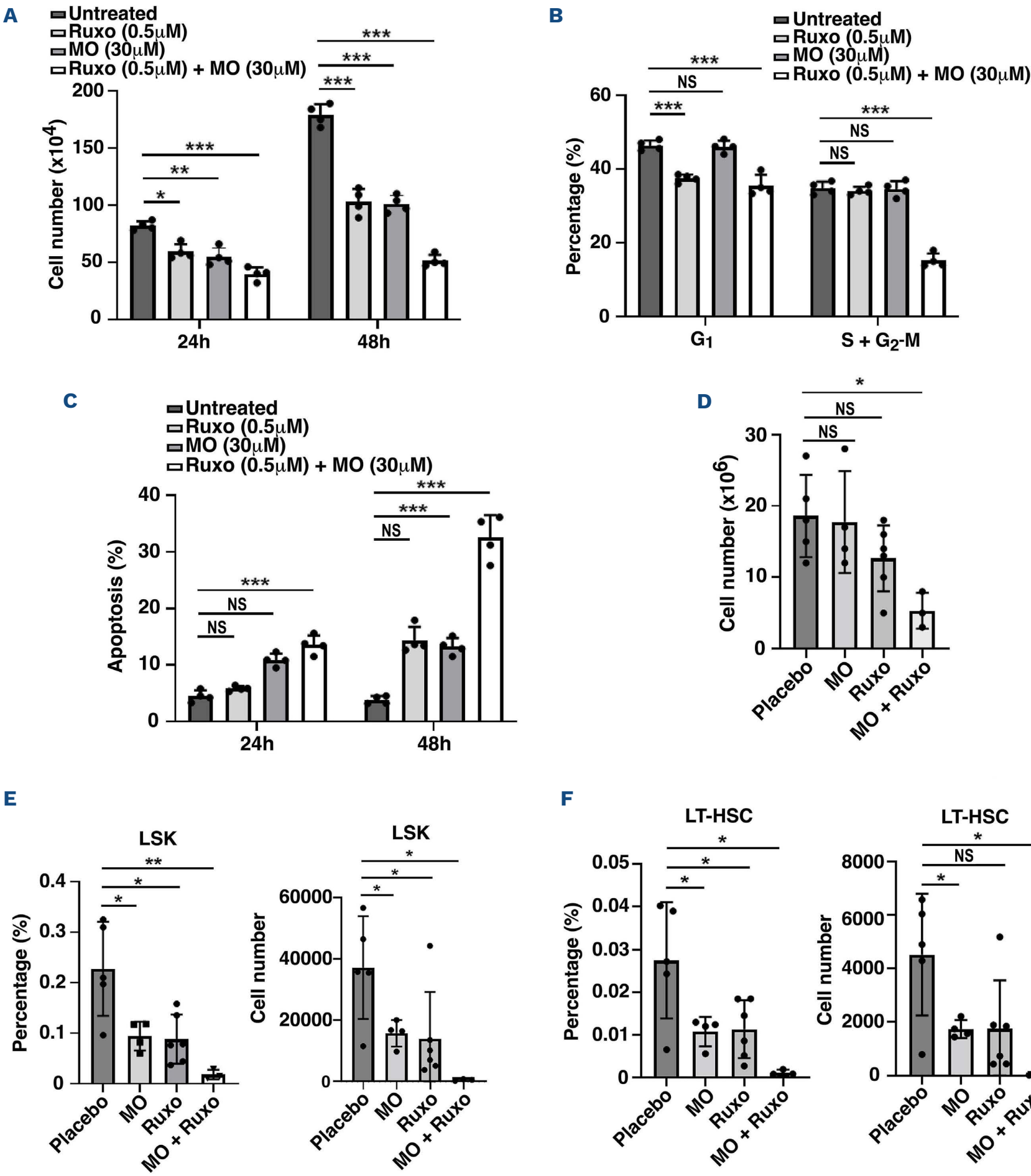

Figure 5. Montelukast synergizes with Ruxolitinib in suppression of polycythemia vera cells. (A) Human HEL cells were treated with Montelukast (MO) alone (30 μM), Ruxolitinib (Ruxo) alone (0.5 μM), or both for 24 and 48 hours, respectively. Cell growth was evaluated by counting live cells (*P<0.05; **P<0.01). (B) HEL cells were treated with Montelukast alone (30 μM), Ruxolitinib alone (0.5 μM), or both for 48 hours, and cell cycle was analyzed by flow cytometry using propidium iodide staining (*P<0.05; **P<0.01). (C) HEL cells were treated with Montelukast alone (30 μM), Ruxolitinib alone (0.5 μM), or both for 24 or 48 hours, and apoptosis of the cells was assessed by flow cytometry using Annexin V/7-aminoactinomycin D staining (*P<0.05; **P<0.01). (D) *JAK2*V617F transgenic mice were treated with a placebo, Montelukast alone (150 mg/kg, once a day), Ruxolitinib alone (90 mg/kg, once a day), or both for 5 months, and total numbers of bone marrow (BM) cells were compared among the treatment groups (D). *JAK2*V617F transgenic mice were treated with a placebo, Montelukast alone, Ruxolitinib alone, or both as described in (D), and the percentage and total number of LSK cells in BM were compared among the treatment groups (E). *JAK2*V617F transgenic mice were treated with a placebo, Montelukast alone, Ruxolitinib alone, or both as described in (D), and the percentage and total number of long-term hematopoietic stem cells ($CD150^+CD48^-c\text{-}Kit^+Sca\text{-}1^+$) in BM were compared among the treatment groups (F). NS: not significant.

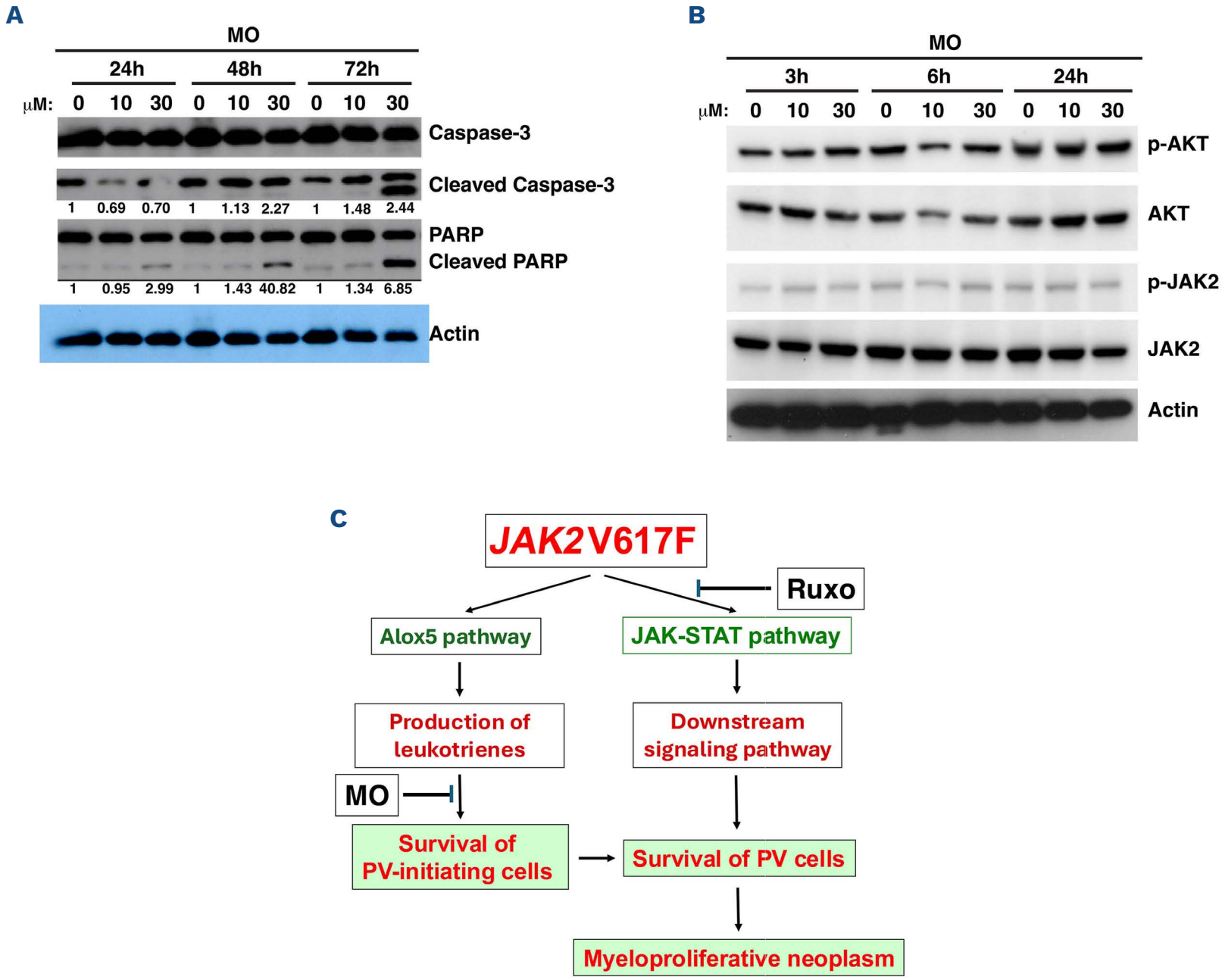

Figure 6. Caspase-3 and PARP are involved in Montelukast-induced apoptosis of *JAK2*V617F-expressing cells. (A) HEL cells were treated with Montelukast (MO) at 10 mM and 30 μM for 24, 48, and 72 hours, respectively. Protein lysates were analyzed by western blotting for expression of precursor and cleaved proteins of Caspase and PARP. Actin was used as a loading control. The levels of cleaved protein expression were quantified by densitometry. (B) HEL cells were treated with MO at 30 μM for 3, 6, and 24 hours, respectively, and protein lysates were analyzed by western blotting for expression of phospho-AKT, AKT, phospho-JAK2, JAK2, and actin. (C) Diagrammatic illustration of a combined targeting strategy for treating human polycythemia vera (PV). Ruxo: Ruxolitinib.

the cleaved Caspase-3 and PARP in Montelukast-treated cells (Figure 6A). We did not detect significant changes in pathway activation of AKT and JAK2 (Figure 6B). These results suggest that apoptosis induced by Montelukast in *JAK2*V617F-expressing cells is mediated by activation of the Caspase-3 and PARP pathways. Considering all results shown in this study, we propose a combined targeting strategy for treating human PV in the future (Figure 6C). To demonstrate that the induction of apoptosis by Montelukast is specific to *JAK2*V617F cells, We treated a non-*JAK2*V617F cell line (BV173, a leukemia cell line that does not express *JAK2*V617F) with or without Montelukast, and did not see an apoptotic effect induced by Montelukast as compared to untreated cells (*Online Supplementary Figure S4*).

Discussion

Transduction of the growth factor-dependent Ba/F3 cell line with the mutant *JAK2* (*JAK2*V617F) facilitates factor-independent survival and proliferation,[10] and mice transplanted with *JAK2*V617F-expressing BM cells develop a PV-like disease.[10,18] These studies indicate that *JAK2*V617F or its downstream signaling molecules could be potential targets for treating PV. We previously showed that, in PV mice, Alox5 expression is increased in *JAK2*V617F-expressing cells and deletion of Alox5 mitigates PV development, suggesting that Alox5 likely functions as a *JAK2*V617F downstream mediator in PV development.[21] However, it is still unclear how Alox5 mediates *JAK2*V617F signaling and plays a critical role in PV development. Leukotrienes, a family of lipid

mediators synthesized by 5-lipoxygenase (5-LO) encoded by the Alox5 gene, are important metabolites produced in the Alox5 pathway that have been reported to play a key role in immune response, inflammatory disorders, and cancer metabolism.[24,26-28,31] Here, we demonstrated that *JAK2*V617F-induced PV responds well to inhibition by Montelukast, a Food and Drug Administration-approved drug for treating human asthma by preventing leukotrienes from binding to their receptors. In other words, as a leukotriene receptor antagonist, Montelukast simply blocks leukotriene function to suppress the Alox5 pathway without affecting the enzymatic activity of 5-LO or Alox5 gene expression. Our results provide a rationale and strategy for using Montelukast to target the Alox5 pathway in PV via blocking the binding of leukotrienes to their receptors.

The identification of *JAK2*V617F mutation deepens our understanding of the molecular pathogenesis of PV. *JAK2*V617F alone is sufficient to induce PV phenotype *in vitro* and *in vivo*,[10,15] showing *JAK2*V617F as a driver mutation in PV and rendering it a logical target for therapeutic intervention. Therefore, the development of PV therapy has been focused on inhibiting *JAK2*V617F kinase activity using an inhibitory compound such as Ruxolitinib, a potent small-molecule inhibitor of JAK1/2 approved by the Food and Drug Administration for treating patients with *JAK2*V617F-associated MPN.[32] Despite the effectiveness of JAK2 inhibitors in treating MPN patients, these drugs do not seem to induce an optimal molecular remission due to their inability to eliminate MPN-initiating cells,[17] a rare hematopoietic population harboring an acquired *JAK2*V617F or other somatic mutations responsible for MPN initiation, progression, and drug resistance.[33] In this study, we show that Montelukast has a significant inhibitory effect on PV-initiating cells, providing a new therapeutic strategy in PV by using a JAK2 inhibitor in combination with Montelukast. On the other hand, Montelukast suppresses the Alox5 pathway by blocking leukotriene-receptor binding with no direct effect on *Alox5* gene expression and JAK2 signaling. We showed in this study that Montelukast acts synergistically with Ruxolitinib in suppressing PV cells. It is necessary to emphasize further that inhibition of *JAK2*V617F kinase activity does not eradicate PV-initiating cells, but inhibition of Alox5 does.[21] We showed that Montelukast suppresses the Alox5 pathway by blocking binding between leukotrienes and their receptors. Thus, inclusion of Montelukast in PV therapy together with other treatment options could be much more effective in treating PV. Based on our work in this study, we propose that a combined therapeutic strategy needs to be tested in human PV patients by targeting PV-initiating cells using a leukotriene inhibitor such as Montelukast and inhibiting more mature PV cells targeting the JAK-STAT pathway using an JAK2 inhibitor such as Ruxolitinib.

It is important to point out that although it effectively suppresses PV-initiating cells, Montelukast does not significantly inhibit normal HSC, indicating that Montelukast preferentially targets PV-initiating cells while sparing their normal stem cell counterparts. By contrast, PV therapy with an JAK2 inhibitor likely has an inhibitory effect on normal HSC, because genetic deletion of the *Jak2* gene in mice leads to severe defects in the functions of normal HSC function.[34,35] Thus, addition of Montelukast to PV therapy with an JAK2 inhibitor would not enhance a potential side effect of the JAK2 inhibitor on normal HSC in treating PV.

Disclosures

No conflicts of interest to disclose.

Contributions

YS and SL conceived the study, conducted experiments, analyzed data, and wrote the manuscript. ND, NL, QQ, KN and YY helped to do the experiments. GM provided the JAK2V617F transgenic mice and advised on the experiments.

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Data-sharing statement

All original data are available upon request to the corresponding author.

References

1. Dameshek W. Some speculations on the myeloproliferative syndromes. Blood. 1951;6(4):372-375.
2. Tefferi A, Vainchenker W. Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. J Clin Oncol. 2011;29(5):573-582.
3. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.
4. Fialkow PJ, Faguet GB, Jacobson RJ, Vaidya K, Murphy S. Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. Blood. 1981;58(5):916-919.
5. Prchal JF, Axelrad AA. Bone-marrow responses in polycythemia vera. N Engl J Med. 1974;290(24):1382.
6. Yumori Y, Sugiyama H, Takahashi T, Haebara H, Hoshino T. [An autopsy case of acute myelodysplasia with myelofibrosis-cytogenetically proved evidence of a clonal disorder with origin in a multipotent stem cell]. Rinsho Ketsueki. 1986;27(4):519-525.
7. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L. Polycythemia vera: stem-cell and probable clonal origin of the disease. N Engl J Med. 1976;295(17):913-916.

8. Cashman JD, Eaves CJ, Eaves AC. Unregulated proliferation of primitive neoplastic progenitor cells in long-term polycythemia vera marrow cultures. J Clin Invest. 1988;81(1):87-91.
9. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365(9464):1054-1061.
10. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434(7037):1144-1148.
11. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352(17):1779-1790.
12. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7(4):387-397.
13. Parganas E, Wang D, Stravopodis D, et al. Jak2 is essential for signaling through a variety of cytokine receptors. Cell. 1998;93(3):385-395.
14. Lu X, Levine R, Tong W, et al. Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. Proc Natl Acad Sci U S A. 2005;102(52):18962-18967.
15. Akada H, Yan D, Zou H, Fiering S, Hutchison RE, Mohi MG. Conditional expression of heterozygous or homozygous Jak2V617F from its endogenous promoter induces a polycythemia vera-like disease. Blood. 2010;115(17):3589-3597.
16. Jamieson CH, Gotlib J, Durocher JA, et al. The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. Proc Natl Acad Sci U S A. 2006;103(16):6224-6229.
17. Mullally A, Lane SW, Ball B, et al. Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential effects on hematopoietic stem and progenitor cells. Cancer Cell. 2010;17(6):584-596.
18. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 2006;107(11):4274-4281.
19. Catalano A, Rodilossi S, Caprari P, Coppola V, Procopio A. 5-Lipoxygenase regulates senescence-like growth arrest by promoting ROS-dependent p53 activation. EMBO J. 2005;24(1):170-179.
20. Chen XS, Sheller JR, Johnson EN, Funk CD. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature. 1994;372(6502):179-182.
21. Chen Y, Shan Y, Lu M, et al. Alox5 blockade eradicates JAK2V617F-induced polycythemia vera in mice. Cancer Res. 2017;77(1):164-174.
22. Markham A, Faulds D. Montelukast. Drugs. 1998;56(2):251-256; discussion 257.
23. Arai Y, Shimoji K, Konno M, et al. Synthesis and 5-lipoxygenase inhibitory activities of eicosanoid compounds. J Med Chem. 1983;26(1):72-78.
24. Stjernschantz J. The leukotrienes. Med Biol. 1984;62(4):215-230.
25. Weller PF, Lee CW, Foster DW, Corey EJ, Austen KF, Lewis RA. Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C4. Proc Natl Acad Sci U S A. 1983;80(24):7626-7630.
26. Romano M, Claria J. Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumor angiogenesis: implications for cancer therapy. FASEB J. 2003;17(14):1986-1995.
27. Runarsson G, Feltenmark S, Forsell PK, Sjoberg J, Bjorkholm M, Claesson HE. The expression of cytosolic phospholipase A2 and biosynthesis of leukotriene B4 in acute myeloid leukemia cells. Eur J Haematol. 2007;79(6):468-476.
28. Vincent C, Fiancette R, Donnard M, et al. 5-LOX, 12-LOX and 15-LOX in immature forms of human leukemic blasts. Leuk Res. 2008;32(11):1756-1762.
29. Zaleskas VM, Krause DS, Lazarides K, et al. Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. PLoS One. 2006;1:e18.
30. Chen Y, Hu Y, Zhang H, Peng C, Li S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet. 2009;41(7):783-792.
31. Sharma JN, Mohammed LA. The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets? Inflammopharmacology. 2006;14(1-2):10-16.
32. Mascarenhas J, Hoffman R. Ruxolitinib: the first FDA approved therapy for the treatment of myelofibrosis. Clin Cancer Res. 2012;18(11):3008-3014.
33. Mead AJ, Mullally A. Myeloproliferative neoplasm stem cells. Blood. 2017;129(12):1607-1616.
34. Akada H, Akada S, Hutchison RE, Sakamoto K, Wagner KU, Mohi G. Critical role of Jak2 in the maintenance and function of adult hematopoietic stem cells. Stem Cells. 2014;32(7):1878-1889.
35. Grisouard J, Hao-Shen H, Dirnhofer S, Wagner KU, Skoda RC. Selective deletion of Jak2 in adult mouse hematopoietic cells leads to lethal anemia and thrombocytopenia. Haematologica. 2014;99(4):e52-54.