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IL-6 stimulation of DNA replication is JAK1/2 mediated in cross-talk with hyperactivated ERK1/2 signaling

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Abstract

Myeloproliferative neoplasms (MPNs) are developing resistance to therapy by JAK1/2 inhibitor ruxolitinib. To explore the mechanism of ruxolitinib's limited effect, we examined the JAK1/2 mediated induction of proliferation related ERK1/2 and AKT signaling by proinflammatory interleukin-6 (IL-6) in MPN granulocytes and *JAK2*V617F mutated human erythroleukemia (HEL) cells. We found that JAK1/2 or JAK2 inhibition prevented the IL-6 activation of STAT3 and AKT pathways in polycythemia vera and HEL cells. Further, we showed that these inhibitors also blocked the IL-6 activation of the AKT pathway in primary myelofibrosis (PMF). Only JAK1/2 inhibitor ruxolitinib largely activated ERK1/2 signaling in essential thrombocythemia and PMF (up to 4.6 fold), with a more prominent activation in *JAK2*V617F positive granulocytes. Regarding a cell cycle, we found that IL-6 reduction of HEL cells percentage in G2M phase was reversed by ruxolitinib (2.6 fold). Moreover, ruxolitinib potentiated apoptosis of PMF granulocytes (1.6 fold). Regarding DNA replication, we found that ruxolitinib prevented the IL-6 augmentation of MPN granulocytes frequency in the S phase of the cell cycle (up to 2.9 fold). The inflammatory stimulation induces a cross-talk between the proliferation linked pathways, where JAK1/2 inhibition is compensated by the activation of the ERK1/2 pathway during IL-6 stimulation of DNA replication.

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Conflict of interest

The authors have declared that no competing interests exist.

Keywords

cell cycle; ERK1/2 signaling; inflammation; JAK1/2 inhibition; myeloproliferative neoplasm

1. Introduction

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders associated with genomic instability, dysregulated signaling pathways and chronic inflammation (Hasselbalch 2012). The most common feature of MPN is acquired somatic *JAK2*V617F mutation that induces constitutive activation of signal transducer and activator of transcription 3 (STAT3) and downstream mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling (Oku et al 2010; Mendoza et al. 2011). We already reported that STAT3 has been significantly increased in *JAK2*V617F homozygous polycythemia vera (PV), besides increased levels of proinflammatory mediator interleukin-6 (IL-6) in plasma and the bone marrow stroma of MPN (Čokić et al. 2015) The constitutive STAT3 activation increases myeloproliferation and inflammation by promoting pro-oncogenic inflammatory pathways, including IL-6 - Janus kinase (JAK) pathway (Oku et al 2010; Reynaud et al. 2011). The *JAK2*V617F presence correlates with increased levels of IL-6 in the plasma of patients with primary myelofibrosis (PMF), associated with shortened survival (Tefferi et al. 2011). Therefore, the inflammatory stimulation of JAK-STAT3 signaling is potentiated by constitutive *JAK2* activation that supports excessive proliferation in MPN.

MPN derived granulocytes, regardless the JAK2 mutational status, are characterized by the upregulation of JAK-STAT target genes, demonstrating the central role of the JAK-STAT pathway in the pathogenesis of MPN (Rampal et al. 2014). A significantly higher phosphorylation of STAT3 in *JAK2*V617F positive MPN patients, which are not dependent on the mutation allele burden, has been demonstrated (Risum et al. 2011). Moreover, JAK2 inhibition reduces STAT3 phosphorylation in PMF granulocytes, and it is also not affected by *JAK2*V617F allele burden (Kalota et al. 2013). In contrast, the gene expression profile is different between *JAK2*V617F-negative and *JAK2*V617F-positive essential thrombocythemia (ET) patients in circulating granulocytes (Schwemmers et al. 2007; Puigdecanet et al. 2008). Furthermore, the constitutive phosphorylation of STAT3 is observed in the neutrophils of MPN patients with high and intermediate *JAK2*V617F mutant allele burden, while the phosphorylation of AKT and MAPK signaling failed to correlate with the *JAK2* mutation status (Mesa et al. 2006). The inhibition of PI3K-AKT signaling prevented the colony formation of *JAK2*V617F-expressing $CD34^+$ cells and hematopoietic progenitors in patients with PMF (Khan et al. 2013). The combination of JAK1/2 and PI3K inhibitors reduced the spleen weight and erythropoietin-independent erythroid colony growth of MPN patients and *JAK2*V617F knock-in mice (Choong et al. 2013). Thus, the constitutive activation of JAK2-STAT3 signaling is supported by the PI3K-AKT pathway.

IL-6 dictates the transition from acute to chronic inflammation by transforming granulocytes to monocytes/macrophages, with a proinflammatory role in the chronic inflammation (Rose-John et al. 2006). We hypothesized that the IL-6 interaction with MAPK and PI3K-AKT

pathways is a potential subsidiary mechanism of the proliferation control in cross-talk with the constitutively activated JAK-STAT signaling in MPN. In accordance with our earlier report, we subsequently analyzed how JAK1/2 mediated the IL-6 regulation of cell proliferation and the activation of STAT3, AKT, and MAPK pathways in the human erythroleukemia (HEL) cellular model and MPN granulocytes.

2. Material and methods

2.1 HEL 92.1.7 and chronic myelogenous leukemia K562 cell lines

HEL 92.1.7 cells with a homozygous expression of *JAK2*V617F, and K562 cells, were cultivated in an RPMI-1640 medium (Biowest, Nuaillé, France) containing 10% fetal bovine serum (FBS, Biowest) and 1% penicillin-streptomycin (Biowest) at 37ºC in a 5% CO_2 humidified atmosphere. According to previous dose dependent studies, 3×10^6 HEL cells were preincubated for 30 minutes with 1 μM ruxolitinib (Cayman chemical company, Ann Arbor, USA) (Heine et al. 2013) or 50 μM 1,2,3,4,5,6-hexabromocyclohexane (hexabromocyclohexane, Sigma-Aldrich, Darmstadt, Germany) (Sandberg et al. 2005), and treated 1 hour with IL-6 (20 ng/ml) (Niemand et al. 2003). Ruxolitinib and hexabromocyclohexane were first dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), but for the applicable final concentration they were dissolved in phosphate-buffered saline (PBS). Parallel with JAK1/2 inhibitors, equal amounts of DMSO were added as a control. For cell cycle analyses, HEL cells were transferred in 2 ml of RPMI-1640 medium (Biowest) in 6-well plates. Seeded HEL cells (starting with 2×10^6 cells) were incubated 16 hours with IL-6 (20 ng/ml), with or without 30 minutes preincubation by ruxolitinib (1 μM - used concentration match to serum level reached in treated MPN patients (Heine et al. 2013)) or hexabromocyclohexane (10 and 50 μM). The viable cell counts were performed with the use of a trypan blue exclusion technique (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Circulating granulocytes of MPN patients

Peripheral blood was obtained from 5 healthy controls and 29 patients diagnosed with MPN according to the World Health Organization (WHO) classification. All of the donors signed the consent form approved by a local ethical committee in accordance with the Declaration of Helsinki. The samples were collected in disodium EDTA and granulocytes were separated using a lymphocyte separation medium (LSM, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and lysing solution (0.15 M NH_4Cl, 0.1 mM Na2EDTA, 12 mM $NaHCO_3$). Besides inverted microscopy (OLYMPUSE PROVIS AX70, Tokyo, Japan), the purity of isolated granulocytes was analyzed (CELLQuest software version 3.0) by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) and proved to be 97% ± 1% CD15 positive (CD15 Monoclonal Antibody, PerCP-eFluor 710, eBioscience™, San Diego, CA, USA). The granulocytes were washed once in PBS and resuspended in RPMI-1640. In order to investigate the signaling pathways linked to JAK2, granulocytes were preincubated for 30 minutes with 1μM ruxolitinib or 50μM hexabromocyclohexane and, subsequently, treated 1 hour with IL-6 (20 ng/ml). For cell cycle analyses, granulocytes (starting with 2×10^6 cells) were incubated 16 hours with IL-6 (20 ng/ml), with or without 30 minutes preincubation by ruxolitinib (1 μM) or hexabromocyclohexane (10 and 50 μM). 10 μM and 50 μM of

hexabromocyclohexane similarly (not significantly different) induced the examined signaling pathways and cell cycle in healthy granulocytes (except ERK1/2, $p<0.01$) and K562 cells, but because 50 μM hexabromocyclohexane kept tendency for more prominent activation we used it in further experiments with IL-6. The viable cell counts were performed with the use of a trypan blue exclusion technique (Gibco, Thermo Fisher Scientific).

2.3 DNA Sequencing

Genomic DNA was extracted from granulocytes of MPN by the proteinase K and phenol-chloroform technique. Single nucleotide mutation *JAK2*V617F was characterized by DNA sequencing after PCR amplification performed with wild-type JAK2-specific forward primer 5′-TGGCAGAGAGAATTTTCTGAACT-3′ and reverse primer 5′-TTCATTGCTTTCCTTTTTCACA-3′. PCR amplified samples were analyzed by sequencing in an automated ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA) with AB DNA Sequencing Analysis Software (v 5.2) by the Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit.

2.4 Immunoblotting

Cells were resuspended in RIPA lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM sodium chloride, 1% Triton x-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 2 mM EDTA and 50 mM sodium fluoride). A protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and sodium orthovanadate (Sigma-Aldrich) were added to the lysis buffer just prior to use. For Western blotting, equal amounts of protein were run on polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Membranes were probed for primary antibodies directed against β-actin (Abcam, Cambridge, UK, ab 1:500), phospho-STAT3/STAT3 (Abcam, cat.no. ab30647/ab137803, 1:500/1:1000), phospho-AKT/AKT (Abcam, cat.no. ab192623/ab179463, 1:1000) and phospho-extracellular signal–regulated kinases 1/2 (ERK1/2, T202/Y204) / ERK1/2 (Cell Signaling Technology Inc., Beverly, USA, cat.no. #9101/9102, 1:1000). Peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnologies, Dallas, USA, cat.no. sc-2054, 1:6000) and goat anti-mouse immunoglobulin (Thermo Scientific, Waltham, USA, cat.no. 31430, 1: 5000) were used as secondary antibodies. Hyperfilm was developed to visualize the secondary antibody by the enhanced chemiluminescence reagent system (GE Healthcare, Amersham, UK) according to the manufacturer's instructions. The phospho-STAT3, phospho-AKT and phospho-ERK1/2 levels were estimated by densitometric scanning of the blots using the Image Master Total Lab (GE Healthcare, Little Chalfont, UK) software tool and normalized to the total STAT3, AKT, and ERK1/2 protein levels, respectively.

2.5 Cell cycle analysis

Following the designated treatments, cell suspensions (1×10^6/0.4 ml PBS containing 2% FBS) were fixed by the drop wise addition of ice-cold 96% ethanol (−20ºC), and then left on ice for at least 30 min. After centrifugation at 1000 rpm/5 min, supernatant was carefully aspirated and cell pellet resuspended. Afterwards, the single-cell suspensions were incubated in a water bath, with 0.5 ml of RNAase solution (1 mg/ml, RNAase A, Thermo Scientific), for 20 min at 37ºC, and then with 0.5 ml of propidium iodide (40 μg/ml in PBS w/o FCS,

Sigma-Aldrich), for 10 min at room temperature in the dark. The cellular DNA content was measured using a CyFlow cytometer (FACSCalibur, BD Biosciences). Usually 3×10^4 cells per sample were analyzed using BDCellQuest Pro software.

2.6 Microarray analysis

For the assessment of mRNA expression levels in $CD34^+$ cells, isolated from peripheral blood, we used biological replicates of: 9 healthy donors, 9 PV, 10 ET, and 4 PMF patients (Čokić et al. 2015). The human oligo probe set used was the Operon Human Genome Array-Ready Oligo Set Version 4.0 (Eurofins MWG Operon, Huntsville, AL, USA) and the RNA samples were processed and analyzed as reported before using total human universal RNA (HuURNA, BD Biosciences, Palo Alto, CA) as a reference in the competitive hybridizations (Čokić et al. 2015). The microarray data obtained are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession no. GSE55976).

2.7 Statistical analysis

The one way ANOVA and Dunnett's posttest were applied using the Prism 4 software tool (GraphPad Software Inc., San Diego, CA, USA). The results are expressed as the mean ± SEM, and p-values < 0.05 are considered to be significant.

3. Results

3.1 JAK-STAT signaling interaction with MAPK and PI3K-AKT pathways in circulatory $CD34^+$ cells of MPN

We already determined increased levels of $CD34^+$ cells and pro-inflammatory cytokine IL-6 in the peripheral blood of MPN (Čokić et al. 2015). Using microarray analyses we observed the gene expression profile related to PI3K-AKT (Table 1) and MAPK (Table 2) pathways in the circulatory $CD34^+$ cells of MPN origin according to the JAK-STAT constitutive activation. Their connection and interference with IL-6 and JAK-STAT pathway is presented in Figure 1. The stimulation of MAPK pathway was supported by PRKCB and RAP1B that stimulated RAF1 expression inhibited by PI3K-AKT pathway (Table 2). Proliferation related RAP1A, MAP2K1 and FOS gene expression were increased predominantly in PV and *JAK2*V617F positive ET (Table 2, Fig. 1). As an inhibitor of the PI3K-AKT pathway, PTEN gene expression was significantly increased in a *JAK2*V617F homozygous form of PV (Table 1). Linked and increased CDKN1B and CCND3 gene expression, related to cell cycle progression, has been observed in MPN (Table 1, Fig. 1). Besides reduced CREB3L4 gene expression, linked and significantly downregulated MCL1 gene expression has been detected in ET, as an apoptosis inducing factor (Table 1, Fig. 1). We observed the gene expression levels linked to PI3K-AKT and MAPK pathways in granulocytes of MPN, but did not find the statistical significance (not shown). Here, we revealed the broad activation of the gene expression levels related to MAPK pathway in $CD34^+$ cells of MPN. In accordance to the low level of $CD34^+$ cells in peripheral blood, we shifted the subsequent protein analyses to granulocytes as the *JAK2*V617F positive or negative myeloid cells.

3.2 Proinflammatory cytokine IL-6 induction of proliferation related signaling pathways in MPN granulocytes

Inflammation biomarkers, such as leukocytes (range 5.2–16.1 × 10^9/l), thrombocytes (321–1059 × 10^9/l), fibrinogen (2.22–5.03 g/l), and erythrocyte sedimentation rate (2–38 mm/h), generally demonstrated a tendency toward elevation in MPN (Table 3). Compared to PV, we observed reduced levels of erythrocyte and hemoglobin in ET and PMF, where *JAK2*V617F negative PMF demonstrated significantly reduced levels of erythrocyte and hemoglobin (Table 3). A marker for the cellular immune system activation, the β2 microglobulin was increased in PV and *JAK2*V617F positive PMF (Table 3). We revealed a continuous IL-6 activation of STAT3 and AKT signaling up to 1 hour in granulocytes of PV and PMF, while dropped below baseline in ET (Fig. 2A, B). IL-6 activation of ERK1/2 signaling was constantly increased in PV and PMF and achieved a plateau after 15 minutes, while the ERK1/2 phosphorylation was transiently increased in ET (Fig. 2C). To examine the *JAK2*V617F mutation dependence, we compared ET and PMF with or without the mutation at different time points during treatment. The *JAK2*V617F presence significantly ($p<0.001$) and steadily increased activation of JAK2-STAT3 pathway in granulocytes of ET during 1 hour of incubation, but not consistent in PMF (Fig. 2D). The *JAK2*V617F mutation transiently upregulated the activation of AKT signaling, peaking at 30 minutes, in the granulocytes of ET and PMF ($p<0.001$, Fig. 2E). The activation of ERK1/2 pathway was also augmented by *JAK2*V617F in PMF, but transiently in ET with the highest difference after 30 minutes ($p<0.001$, Fig. 2F). ERK1/2 phosphorylation was in negative correlation with the leukocyte number in PV ($r=-0.917$, $p=0.083$). In contrast, AKT phosphorylation was in positive correlation with the leukocyte number ($r=0.865$, $p=0.026$) and fibrinogen levels ($r=0.887$, $p=0.018$) in PMF. We presented the increased level of inflammation biomarkers and time dependent activation of JAK-STAT3, AKT, and ERK1/2 pathways by proinflammatory IL-6, affected by *JAK2*V617F mutation status in MPN.

3.3 JAK1/2 mediated regulation of JAK2-STAT3 signaling pathway by IL-6 in MPN granulocytes

JAK1/2 inhibitor ruxolitinib and specific JAK2 inhibitor hexabromocyclohexane prevented IL-6 activation of JAK2-STAT3 pathway in the granulocytes of PV, after 1 hour of incubation ($p<0.001$, Fig. 3A). Contrary, IL-6 inhibited STAT3 activation in ET granulocytes regardless the *JAK2*V617F mutation status (Fig. 3B, C). The JAK1/2 and JAK2 inhibitors blocked the IL-6 reduction of JAK2-STAT3 signaling in *JAK2*V617F positive granulocytes of ET, while the absence of the mutation enhanced inhibition of JAK2-STAT3 signaling (1.75 fold by ruxolitinib, Fig. 3C). The used concentration of ruxolitinib did not prevent the constitutive STAT3 phosphorylation in the granulocytes of *JAK2*V617 positive ET, while ruxolitinib and hexabromocyclohexane reduced the phosphorylation of STAT3 in *JAK2*V617 negative ET (Fig. 3B, C). IL-6 activation of JAK-STAT3 pathway (1.6 fold) was supported by hexabromocyclohexane in *JAK2*V617F mutated PMF granulocytes (Fig. 3D). Furthermore, JAK1/2 and JAK2 inhibitors failed to block the constitutive activation of JAK-STAT3 signaling in PMF (Fig. 3D, E). IL-6 activated JAK2-STAT3 signaling in PV and PMF, but the activation only demonstrated JAK1/2 dependence in PV.

3.4 JAK1/2 mediated regulation of AKT signaling pathway by IL-6 in MPN granulocytes

Both JAK1/2 and JAK2 inhibitors blocked the IL-6 activation of AKT signaling (1.4 fold) in *JAK2*V617F-positive HEL cells (Fig. 4A). IL-6 enhanced phosphorylation of AKT pathway was prevented only by specific JAK2 inhibitor hexabromocyclohexane in PV granulocytes (Fig. 4B). IL-6 reduced AKT phosphorylation in ET was reversed and stimulated by JAK1/2 and JAK2 inhibitors (up to 1.7 fold) in *JAK2*V617F mutated ET granulocytes (Fig. 4C, D). Hexabromocyclohexane and ruxolitinib also reduced AKT phosphorylation in *JAK2*V617F negative ET (Fig. 4D). IL-6 activation of AKT signaling (1.4 fold) was blocked by both JAK1/2 and JAK2 inhibitors in PMF (Fig. 4E, F). IL-6 failed to induce AKT signaling in granulocytes of healthy controls and K562 cells, while ruxolitinib (1.4 fold) activated in K562 cells and hexabromocyclohexane (1.3 fold) in granulocytes (not shown). IL-6 activation of AKT signaling is JAK1/2 dependent in HEL cells and granulocytes of PV and PMF.

3.5 JAK1/2 mediated regulation of MAPK signaling pathway by IL-6 in MPN granulocytes

IL-6 enhanced phosphorylation of ERK1/2 pathway in granulocytes of healthy subjects only in co-treatment with hexabromocyclohexane ($p<0.001$, 1.44 fold, Fig. 5A). IL-6 enhanced phosphorylation of the ERK1/2 pathway, in PV granulocytes, was not affected by JAK1/2 and JAK2 inhibitors (Fig. 5B). IL-6 activated ERK1/2 signaling only in *JAK2*V617 negative ET (Fig. 5C, D). Moreover, ruxolitinib largely stimulated ERK1/2 signaling in ET (up to 6.5 fold) regardless the *JAK2*V617F presence, compared to modest hexabromocyclohexane increase (Fig. 5C, D). IL-6 activation of ERK1/2 signaling was enhanced by non-specific JAK1/2 inhibitor ruxolitinib (up to 1.6 fold), reduced by specific JAK2 inhibitor hexabromocyclohexane (up to 1.7 fold), and not affected by *JAK2*V617F mutation status in PMF granulocytes (Fig. 5E, F). Individually, IL-6 (1.46 fold) and hexabromocyclohexane (1.7 fold) activated ERK1/2 signaling in K562 cells (not shown), but not in granulocytes of healthy controls (Fig. 5A). Moreover, hexabromocyclohexane supported IL-6 activation of ERK1/2 signaling in K562 cells (1.6 fold, not shown). Ruxolitinib activated largely ERK1/2 signaling in the granulocytes of ET and PMF patients.

3.6 JAK1/2 mediated regulation of cell cycle induced by IL-6

To study the JAK2-STAT3 mediated IL-6 induction of myeloproliferation, we demonstrated that IL-6 with or without JAK1/2 inhibitors double increased apoptosis and 1.5 fold reduced granulocyte frequency of G0/G1 phase in K562 cells (Fig. 6A). Ruxolitinib reversed IL-6 reduction of HEL cells quantity (as a cell proliferation model) in G2M phase of cell cycle after 16 hours of incubation ($p<0.05$, Fig. 6B). S phase was not significantly changed by IL-6 and JAK1/2 inhibitors in granulocytes of healthy donors (Fig. 6C). Regarding the S and G2/M phases, the highest quantity of positive granulocytes was observed for PV (Fig. 6D). The thrombocyte quantity was in positive correlation with the PV granulocytes arrested in S phase of cell cycle ($r=0.997$, $p=0.003$). The highest number of granulocytes in G0/G1 phase was observed in ET (Fig. 6E), especially *JAK2*V617F negative ET (not shown). The G0G1 portion of granulocytes was significantly reduced in PMF by IL-6 regardless JAK1/2 inhibition ($p<0.01$, Fig. 6F). In addition, IL-6 largely increased the percentage of ET- (2.2 fold, $p<0.001$) and PMF- (2.8 fold, $p<0.001$) derived granulocytes in S phase of cell cycle

that was completely abolished by JAK1/2 and JAK2 inhibitors (Fig. 6E, F). Regarding cell survival, both ruxolitinib and hexabromocyclohexane with IL-6 increased apoptosis of PMF granulocytes (up to 1.6 fold, $p<0.05$, Fig. 6F). JAK1/2 and JAK2 inhibitors reduced apoptosis in *JAK2*V617F positive, but increased in *JAK2*V617F negative granulocytes of PMF patients ($p<0.01$, not shown). IL-6 stimulation of DNA replication is JAK1/2 mediated in granulocytes of MPN.

4. Discussion

JAK2-STAT3 signaling activated the PI3K/AKT and MAPK pathways (Araki 2016). As major regulators of cell survival and proliferation, the MAPK and PI3K-AKT pathways were activated during oncogenesis due to their cross-activation and pathway convergence that can facilitate the acquisition of resistance to therapeutics targeting only one pathway (Mendoza 2011). IL-6 stimulated MAPK, PI3K-AKT and via IL-6 receptor STAT3 phosphorylation, while the last one was not prevented by MAPK and PI3K signaling inhibition (Hideshima et al. 2001; Fahmi et al. 2013). IL-6 induced MAPK phosphorylation was partially blocked by PI3K signaling inhibition, while PI3K-AKT phosphorylation was not prevented by MAPK signaling inhibition (Hideshima et al. 2001). We observed that IL-6 increased phosphorylation of ERK1/2 signaling was prevented by specific JAK2 inhibition in PMF granulocytes. However, only non-specific JAK1/2 inhibition largely stimulated ERK1/2 signaling in granulocytes of ET and PMF patients.

A difference in the molecular mechanism of inhibition between the JAK1/2 inhibitor ruxolitinib and the specific JAK2 inhibitor hexabromocyclohexane can explain this paradox. Hexabromocyclohexane reduced JAK2 tyrosine kinase autophosphorylation by inhibiting ligand dependent activation, while ruxolitinib acted as an ATP-competitive inhibitor of JAK1/2 kinase activation of downstream pathways (Sandberg et al. 2005; Quintás-Cardama et al. 2010) The persistence of JAK2 inhibition was associated with the reversible activation of JAK–STAT signaling and heterodimerization between the activated JAK2 and JAK1 or TYK2, consistent with the activation of JAK2 in trans by other JAK kinases in MPN (Koppikar et al. 2012). Furthermore, JAK1/2 inhibition can potentiate the TYK2-dependant activation of ERK1/2, while the specific inhibition of JAK2 may destabilize a complex with IL-6 receptor diminishing ERK1/2 activation.

PI3K inhibition blocked IL-6-induced G1/S phase transition and G1 growth arrest (Hideshima et al. 2001). IL-6 induction of G1-arrested cells was sufficient to induce S phase entry and prevent apoptosis (Côté et al. 2005). IL-6 increased percentage of cells in S phase through the activation of JAK2-STAT3 and ERK1/2 pathways (Suh et al. 2008). Activated STAT3 increased proliferation and tumor-promoting inflammation, while also inhibited apoptosis (Klein et al. 2014; Yu et al. 2009). In contrast, the parallel inhibition of IL-6/STAT3 and ERK1/2 pathways led to strong induction of apoptosis (Chatterjee et al. 2004). We also demonstrated that pro-inflammatory IL-6 generally increased percentage of MPN granulocytes in S phase, through JAK1/2 control, promoting cell proliferation.

In vitro proliferation of erythroid cells from *JAK2*V617F-positive MPN patients was synergistically regulated by STAT, PI3K/AKT and MAPK pathways, while PI3K inhibition

blocked MAPK activation (Wolf et al. 2013). An increased phosphorylation of STAT3 in the *JAK2*V617F positive MPN patients, with no positive correlation with *JAK2*V617F allele burden has been reported (Risum et al. 2011). Moreover, the efficacy of ruxolitinib was irrelevant to the *JAK2*V617F mutation status in patients with PMF (Chen et al. 2016). In addition, an increased phosphorylation of STAT3 was not affected by *JAK2*V617F mutation status in the bone marrow of PV and ET (Teofili et al. 2007). The deletion of Stat3 slightly reduced the erythrocytes and hematocrit parameters, increased platelets and neutrophils, and markedly reduced the survival of *Jak2*V617F knock-in mice (Yan et al. 2015; Grisouard et al. 2015). According to the presented results, an overview of the JAK1/2 mediated IL-6 induction of the proliferation related pathways is shown in Table 4. Furthermore, it has been reported that ruxolitinib reduced constitutive STAT3 phosphorylation with a modest effect on AKT signaling in the *JAK2*V617F mutated HEL cell line (Quintás-Cardama et al. 2010). Another study showed that the PI3K/AKT pathway is involved in a malignant transformation by *JAK2*V617F mutation (Kamishimoto et al. 2011). We demonstrated that IL-6 inhibition of AKT signaling is overturned by both JAK2 and JAK1/2 inhibitors in the *JAK2*V617F positive ET granulocytes. Contrarily, IL-6 activation of AKT signaling is prevented by JAK2 and JAK1/2 inhibition in PMF, regardless of the *JAK2*V617F status.

The PI3K/AKT/mTOR inhibition induced a cell-cycle growth arrest and apoptosis of the primary $CD34^+$ MPN cells, synergistically supported by JAK2 inhibition, while sparing the normal $CD34^+$ cells (Fiskus et al. 2013). Moreover, PI3K/mTOR inhibitor in combination with the JAK1/2 inhibitor ruxolitinib prolonged survival and improved clinical parameters in conditional *Jak2*V617F knock-in mice (Bartalucci et al. 2013). In a Phase I/II trial with mTOR inhibitor everolimus, 60% of PMF patients showed improvement in constitutional symptoms and a decrease in spleen enlargement although to a lesser extent than JAK inhibitors, with no changes in mutant allele burden and cytokine profile (Guglielmelli et al. 2011). Therefore, the PI3K/AKT/mTOR signaling pathway is an auxiliary structure for the regulation of proliferation in MPN.

5. Conclusions

IL-6 induced DNA replication in granulocytes, thus preventing apoptosis via the JAK1/2 stimulation in MPN. IL-6 stimulated JAK2-STAT3 and AKT signaling in PV and PMF, but reduced in ET. JAK1/2 inhibition largely stimulated MAPK signaling in ET and PMF, regardless *JAK2*V617F mutation. The treatment that integrates the inhibition of JAK2-STAT3 and MAPK pathways may be crucial to overcome the cross-activation responsible for limitations in the current targeted therapy of MPN.

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Abbreviations

DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinases
ET	essential thrombocythemia
FCS	fetal calf serum
HEL	Human erythroleukemia cells
JAK2	Janus kinase 2
MAPK	Mitogen Activated Protein Kinases
MPN	myeloproliferative neoplasm
PI3K	phosphatidylinositol 3-kinase
PMF	primary myelofibrosis
PV	polycythemia vera
STAT	Signal Transducer and Activator of Transcription

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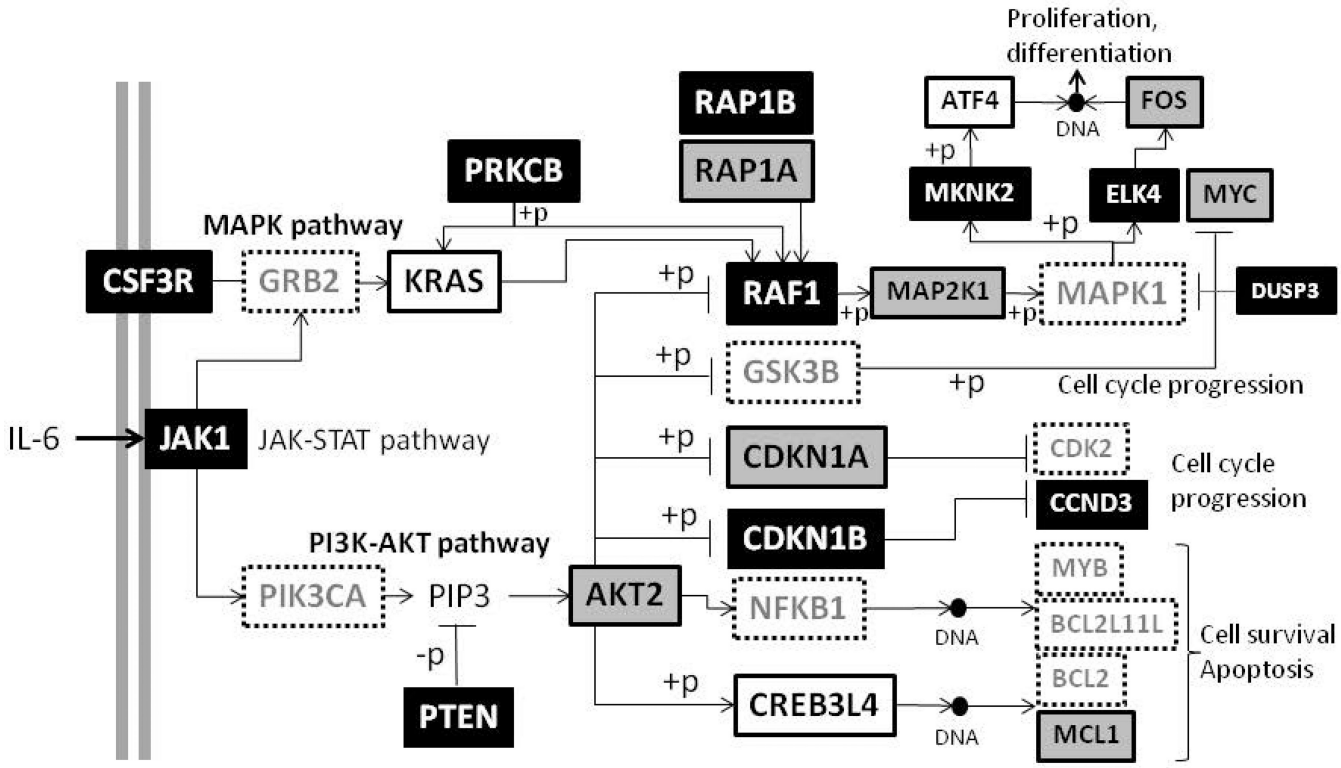

Figure 1.

JAK-STAT signaling stimulation of MAPK and PI3K/AKT pathways in CD34^{+} cells of MPN origin (correspond to Tables 1 and 2). (+p) phosphorylation, (-p) dephosphorylation; → stimulation, ⊥ inhibition; white boxes represent down-regulated genes, gray boxes mixed gene expression, black boxes up-regulated genes *vs.* controls, while white boxes with an intermittent edge represent sporadically expressed genes in MPN *vs.* control.

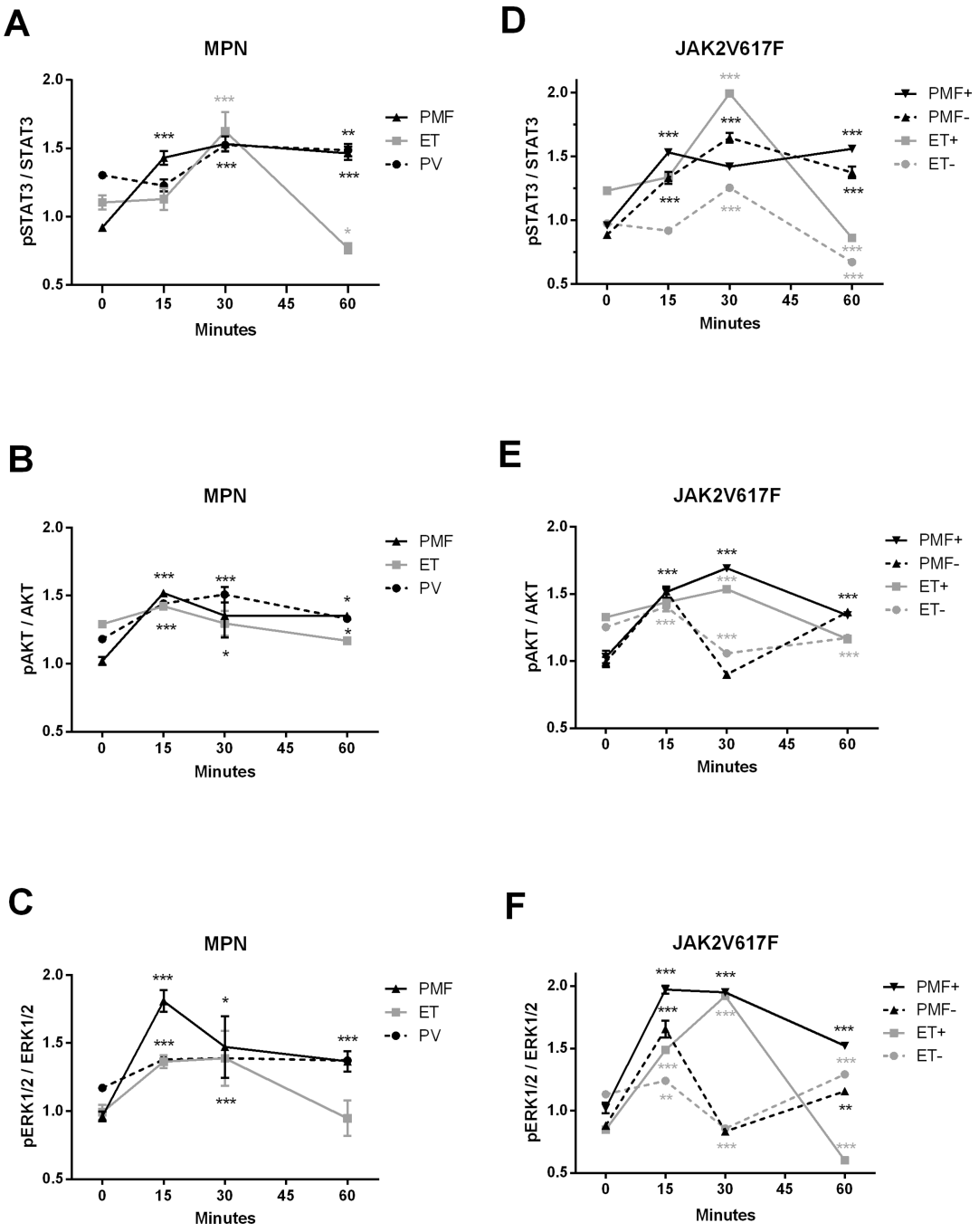

Figure 2.

Activation of proliferation related signaling pathways by IL-6 in MPN granulocytes. Time scale of IL-6 (20 ng/ml) activation of (A) JAK/STAT3; (B) AKT and (C) ERK1/2 signaling in granulocytes of polycythemia vera (PV, n = 6), essential thrombocythemia (ET, n = 8) and primary myelofibrosis (PMF, n = 8). *p < 0.05, **p < 0.01, ***p < 0.001 vs. non-treated cells (0). IL-6 activation of (D) JAK/STAT3; (E) AKT and (F) ERK1/2 signaling in ET and PMF according to *JAK2*V617F presence during 1 hour of incubation. (n = 4) Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs. non-mutated cells.

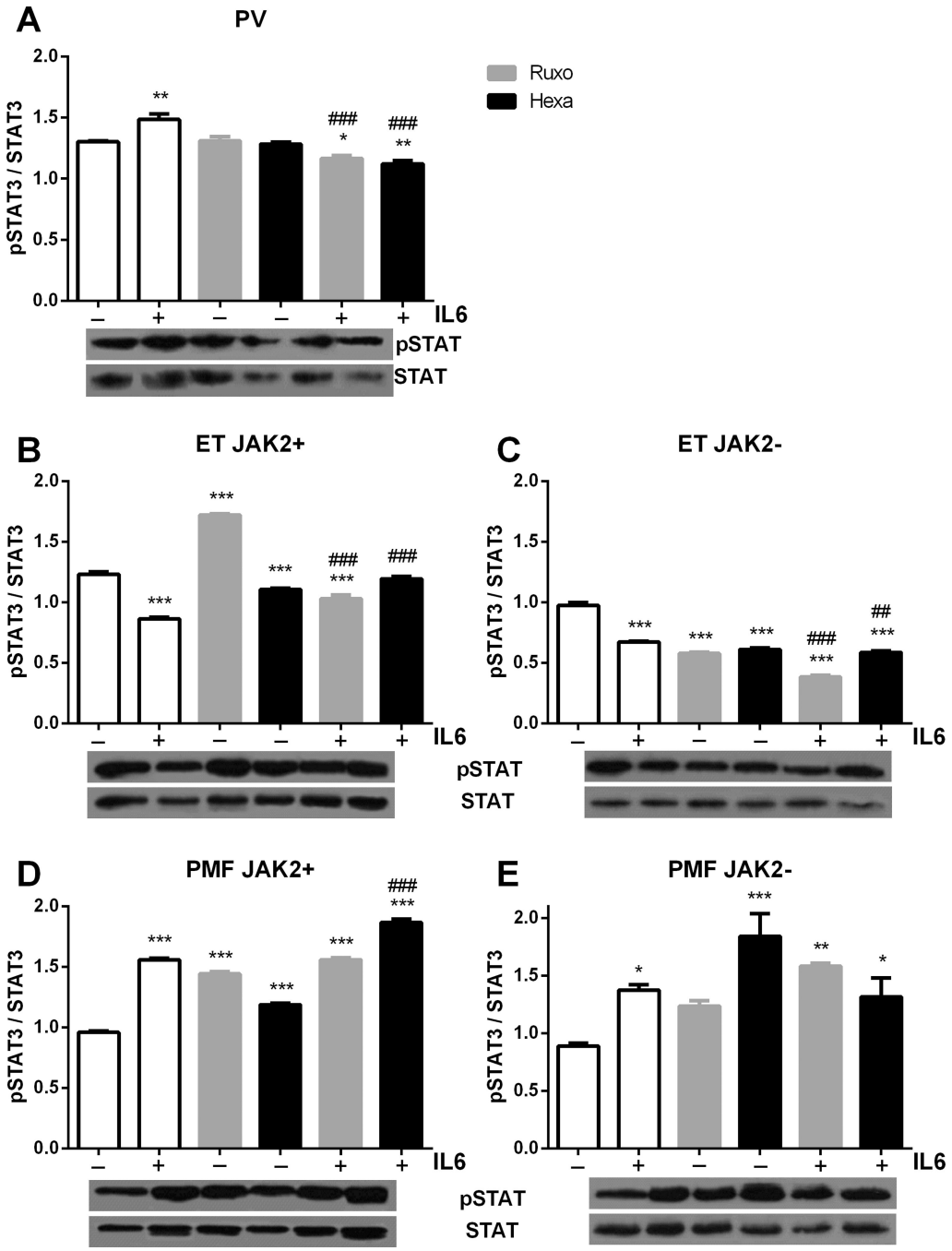

Figure 3.

IL-6 activation of STAT signaling in PV and PMF as well as inhibition in ET granulocytes. IL-6 (20 ng/ml) activation of JAK/STAT3 signaling in (A) polycythemia vera (PV), (B) essential thrombocythemia (ET) with *JAK2*V617F (JAK2+), (C) ET without *JAK2*V617F (JAK2-), (D) primary myelofibrosis (PMF) JAK2+, (E) PMF JAK2-, in the presence of ruxolitinib (Ruxo) and hexabromocyclohexane (Hexa) after 1 hour of incubation. Values are mean ± SEM (n=4). *p < 0.05, **p < 0.01, ***p < 0.001 vs. non-treated cells/Control; #the same vs. IL-6 treated cells.

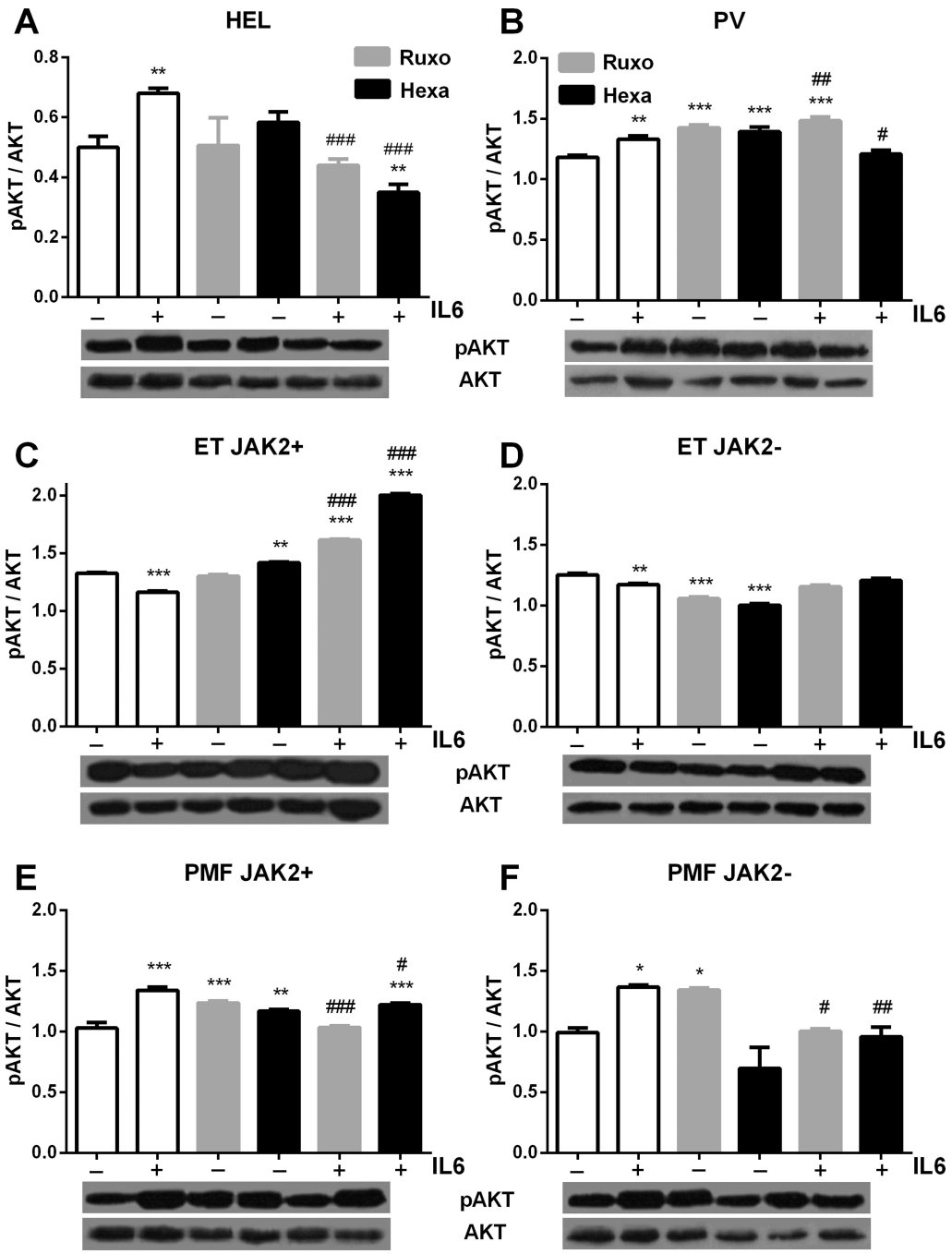

Figure 4.

JAK1/2 dependent activation of AKT pathway by IL-6 in PV and PMF besides inhibition in ET granulocytes. IL-6 (20 ng/ml) activation of AKT signaling in (A) HEL cells, (B) polycythemia vera (PV), (C) essential thrombocythemia (ET) with *JAK2*V617F (JAK2+), (D) ET without *JAK2*V617F (JAK2-), (E) primary myelofibrosis (PMF) JAK2+, (F) PMF JAK2-, in the presence of ruxolitinib (Ruxo) and hexabromocyclohexane (Hexa) after 1 hour of incubation. Values are mean ± SEM (n=4). *p < 0.05, **p < 0.01, ***p < 0.001 vs. non-treated cells/Control; #the same vs. IL-6 treated cells.

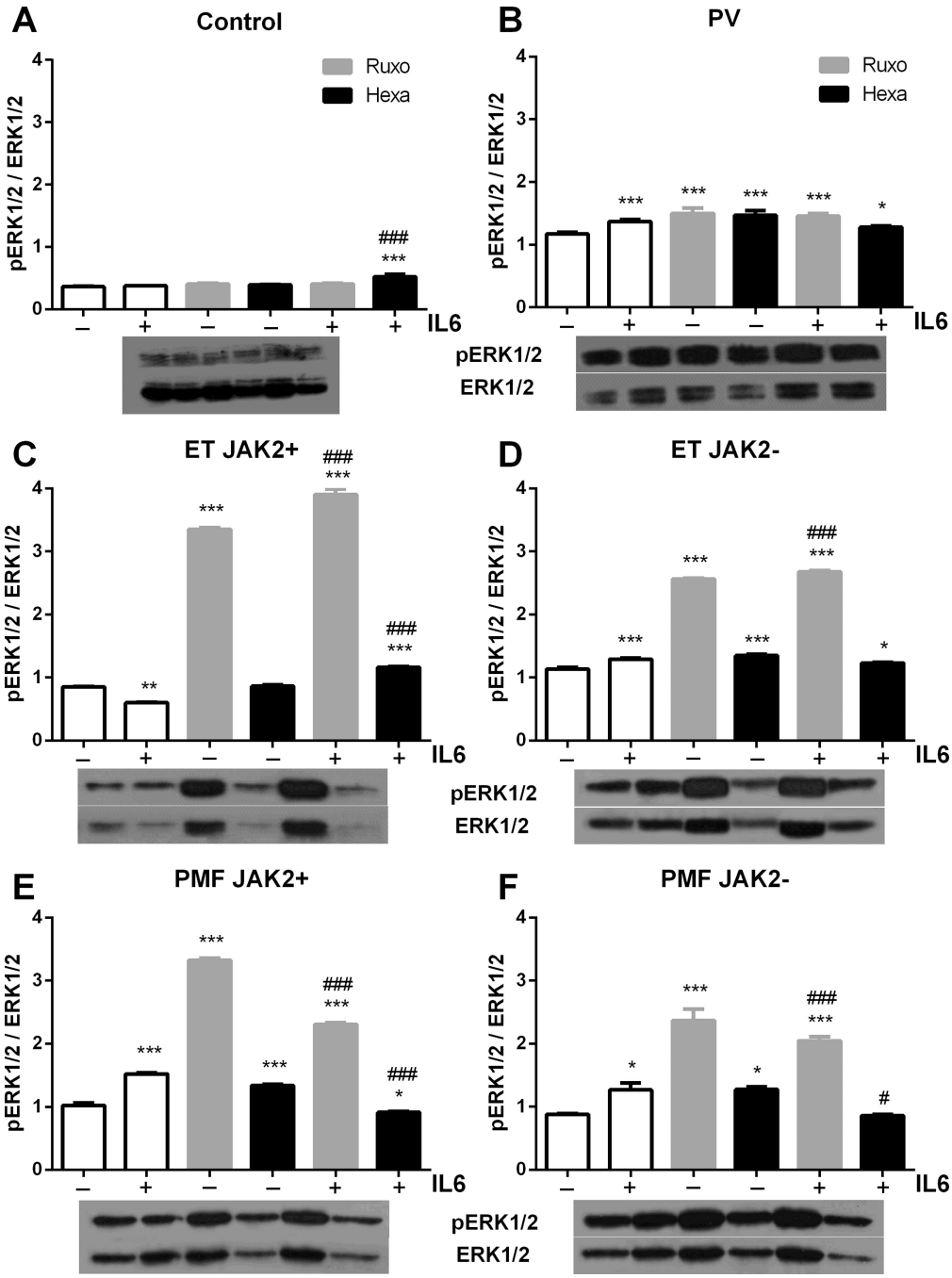

Figure 5.

IL-6 activation of MAPK pathway in PV and PMF then inhibition in JAK2+ ET granulocytes. IL-6 (20 ng/ml) activation of ERK1/2 signaling in (A) healthy controls, (B) polycythemia vera (PV), (C) essential thrombocythemia (ET) with *JAK2*V617F (JAK2+), (D) ET without *JAK2*V617F (JAK2-), (E) primary myelofibrosis (PMF) JAK2+, (F) PMF JAK2-, in the presence of ruxolitinib (Ruxo) and hexabromocyclohexane (Hexa) after 1 hour of incubation. Values are mean ± SEM (n=4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. non-treated cells/Control; $^{###}p < 0.001$ vs. IL-6 treated cells.

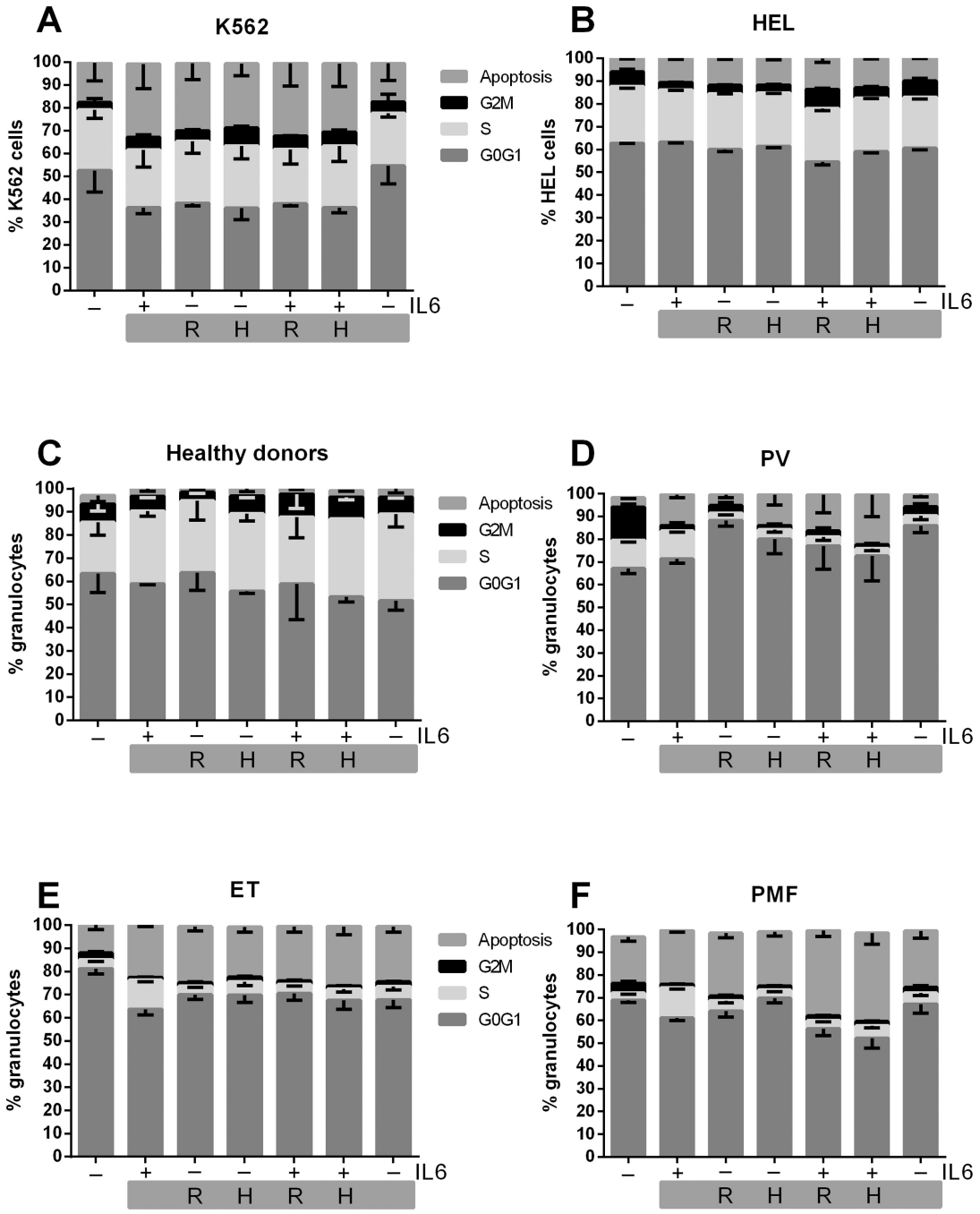

Figure 6.

IL-6 increased the percentage of MPN granulocytes in S phase of cell cycle mediated by JAK1/2. IL-6 (20 ng/ml) induction of cell cycle in (A) K562 cells (n=3), (B) *JAK2*V617F mutated HEL cells (n=3), (C) healthy donors (n=3), (D) polycythemia vera (PV, n=4), (E) essential thrombocythemia (ET, n=8) and (F) primary myelofibrosis (PMF, n=8) treated with Ruxolitinib (R) or Hexabromocyclohexane (H) after 16 hours of incubation (gray box). Values are mean±SEM.

Table 1:

PI3K/AKT pathway related gene expression in circulatory $CD34^+$ cells of MPN origin.

Genes	Full name of genes	Control (n=9)	PV htz (n=4)	PV hom (n=5)	ET htz (n=6)	ET no mut (n=4)	PMF (n=4)
CSF3R	colony stimulating factor 3 receptor	2.8±0.2	3.2±0.6	3.2±0.2	3.8±0.6**	3±0.4	3.1±0.8
CDK4	cyclin-dependent kinase 4	−0.9±0.3	−1.8±0.3	−1.5±0.7	−1.8± 0.3**	−1.7±0.2	
CCND3	cyclin D3	0.2±0.2	0.7±0.6	0.9±0.3	1.1±0.5	0.9±0.4**	1±0.4**
PPP2R5C	protein phosphatase 2, regulatory subunit B, gamma	−0.6±0.2	−0.4±0.2	−0.1±0.5	−0.1±0.2**	−0.4±0.1	−0.5±0.4
MCL1	myeloid cell leukemia sequence 1	3±0.6	1.7±1	3.2±0.3	1.9±0.6	2.6±0.2*	2±0.8
HSP90AB1	heat shock protein 90kDa alpha B1	−0.9±0.4			−1.6±0.3	−1.6±0.5*	−0.6±0.5*
FGFR4	fibroblast growth factor receptor 4	1.9±0.2	2.3±0.7	3.2±0.3**	2.4±0.8	2.6±0.1	2.5±0.7
JAK1	Janus kinase 1	0.9±0.2	1.4±0.03	1.7±0.1**	0.9	1.2	1.3±0.2
YWHAH	tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein, eta polypeptide	0.2±0.5	1.2±0.3**	1±0.4	1.1±0.8	1.2±0.7	1.4±0.3**
YWHAQ	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, theta polypeptide	−0.5±0.9	−0.03±0.4	0.1±0.4	−0.6±0.5	−0.3±0.3	0.6±0.6
YWHAB	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein,β-polypeptide	−0.4±0.4	0.5±0.5*	−0.1±0.2	−1.6±0.4	−1.3±0.2	−0.3±0.5
PTEN	phosphatase and tensin homolog	2.1±0.6	3±0.3	3.1±0.2*	2.9±0.6	3.1	2.6±0.7
COMP	cartilage oligomeric matrix protein	0.7±0.2	−0.4±0.1*	−0.1			
FN1	fibronectin 1	−3.2±0.4	−4.2±0.1*	−4.1	−4±0.7	−3	−3.8±0.3**
STK11	serine/threonine kinase 11	1.8±0.4	2.5±0.4*	2.1±0.3	2.5±1	1.4±0.7	1.7±0.8
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.7±0.1		1.3±0.1*		0.3±0.8	1.3
PGF	placental growth factor	−2.5±0.9	−3.1±0.3	−3.8±0.4	−2.6±0.6	−2.9±0.9	−3.4±0.7
ITGB7	integrin, beta 7	−1.5±0.6	−1.7±0.5	−2±0.6	−2±0.5	−2.1±0.2	−2.3±0.3*
ATF4	activating transcription factor 4	0.1±0.2	−0.6±0.1*	−0.4±0.4	−0.2	−0.1±0.3	−0.1±0.2
GNG12	guanine nucleotide binding protein	0.3±0.6	0.3±0.4	−0.5±0.3*	−0.1±0.4	0.3±0.2	−0.1±0.2

Genes	Full name of genes	Control (n=9)	PV htz (n=4)	PV hom (n=5)	ET htz (n=6)	ET no mut (n=4)	PMF (n=4)
MYB	v-myb myeloblastosis viral oncogene homolog	0.9±0.3	0.5	1.9			2.4±0.3*
CDC37	cell division cycle 37 homolog	0.8±0.2	0.3±0.1	0.6±0.2	0.7±0.3	0.8±0.5	0.4±0.2*
PIK3CA	Phosphoinositide-3-kinase, catalytic, α polypeptide		1.5	1.6±0.1	1.4		
AKT2	v-akt murine thymoma viral oncogene homolog 2	0.9±0.2	0.7±0.4	1.3±0.1	0.8±0.4	1.1±0.6	0.9±0.3
GSK3B	glycogen synthase kinase 3β	1.5±0.6	1.7	0.9±0.3	0.8±0.7	1.3	0.9±0.2
CDKN1A	cyclin dependent kinase inhibitor 1A	2.4±0.6	2.5±0.9	1.8±0.6	2.7±1.5	2.1	2.2±0.3
CDKN1B	cyclin-dependent kinase inhibitor 1B	1.1±0.9	1.8±0.4	2±0.6	1.4±0.6	1.1±0.7	1.5±0.8
CDK2	cyclin-dependent kinase 2	−0.8±0.2					0.6
RBL2	retinoblastoma-like 2	2.4±0.5	2.4±0.2	2.2±0.3	2.7±0.6	2.3±0.1	1.9±0.6
CREB3L4	cAMP responsive element binding protein 3-like 4	2.6±0.7	2.1±0.2	1.9±0.5	1.6±0.5	1.5±0.2	1.3
BCL2L11	BCL2-like 11 (apoptosis facilitator)	1.4±0.03					1.2
BCL2	B-cell CLL/lymphoma 2	0.7	1.1		1.3		
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	3±0.6	1.7±1	3.2±0.3	1.9±0.6	2.6±0.2*	2±0.8

polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF)

** p<0.01

* p<0.05 vs. Control

htz-heterozygosity, hom – homozygosity, no mut – no mutation

Table 2:

MAPK signaling pathway related gene expression in circulatory $CD34^+$ cells of MPN origin.

Genes	Full name of genes	Control (n=9)	PV htz (n=4)	PV hom (n=5)	ET htz (n=6)	ET no mut (n=4)	PMF (n=4)
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	0.6±0.7	0.5±0.6	1.8±0.5*	1.8±0.5*	0.5±0.6	1.6±0.4*
DUSP3	dual specificity phosphatase 3	−0.5±0.1	−0.4±0.3	−0.4±0.2	−0.2±0.1	−0.5±0.1	−0.3±0.1
DUSP6	dual specificity phosphatase 6	2.1±0.4	3.3±0.6*	2.9±0.5	3.3±0.8*	3.2±0.7	2.7±0.9
GADD45B	growth arrest and DNA-damage-inducible, beta	1.3±0.4	1.4±0.3	0.7±0.5	2.2±0.7*	1.5±0.7	0.2±1*
RAC3	ras-related C3 botulinum toxin substrate 3	1.5±0.1	1.5±0.01	1.3±0.2	1.1±0.1*	1.1	1.2±0.2
HSPA8	heat shock 70kDa protein 8	−1±0.6	−0.4±0.4	0.5±0.5**	−0.6±0.5	−0.8±0.2	−0.3±0.6
FGFR4	fibroblast growth factor receptor 4	1.9±0.2	2.2±0.7	3.2±0.3**	2.4±0.8	2.6±0.1	2.5±0.7
MAP3K4	Mitogen-activated protein kinase kinase kinase 4	−2.3±0.2	−3.3±0.3	−4.1±0.04**	−3.8		−3.3± 0.4
MAPK8IP2	mitogen-activated protein kinase 8 interacting protein 2	0.5±0.7	−0.1±0.4	−0.7±0.3*	0.1±0.7	0.6±0.5	−0.6±0.4*
ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)	1.1±0.5	2±0.4	2.3±0.5*	1.9±0.6	1.9±0.4	1.4±0.3
CACNA1S	calcium channel, voltage-dependent, L type, alpha 1S subunit	−1.5±0.8	−2.6± 0.6*	−2.5± 0.2	−2.2± 0.8	−2.1±1.3	−2.3±0.8
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.7±0.1		1.3±0.1*		0.3±0.8	1.3
HSPA1A	heat shock 70kDa protein 1A	−0.4±0.5	1.1±0.4*	0.9±1.2	0.3±0.6	−0.3±0.6	0.2±0.4
RAC2	ras-related C3 botulinum toxin substrate 2	2.7±0.6	3.6±0.3*	3.3±0.2	3.2±0.5	3.2±0.5	3.2±0.3
RAP1A	RAP1A, member of RAS oncogene family	0.9±0.4	1.3±0.5	1.5±0.4	1.3±0.2	0.7±0.4	0.8±0.5
RAP1B	RAP1B, member of RAS oncogene family	0.7±0.5	1.2±1.2	1.9±0.4*	1.3±1	1.7±0.5	2.2±0.5**
FOS	FBJ murine osteosarcoma viral oncogene homolog	2.5±0.9	4.1±0.3*	3.4±1.1	3.6±0.8	2.3±0.8	2.4±1
MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.6±0.2	1.6±0.4*	0.7±0.2	0.8±0.5	0.2	0.3
ATF4	activating transcription factor 4	0.1±0.2	−0.6± 0.1*	−0.4± 0.4	−0.2	−0.1±0.3	−0.1±0.2
GNG12	guanine nucleotide binding protein, gamma 12	0.3±0.6	0.3±0.4	−0.5± 0.3*	−0.1±0.4	0.3±0.2	−0.1±0.2
PRKCB	protein kinase C, beta	1.8	2.6±0.03	2.3±0.4	2.3±0.4	2.3±0.4	1.4±0.9

Genes	Full name of genes	Control (n=9)	PV htz (n=4)	PV hom (n=5)	ET htz (n=6)	ET no mut (n=4)	PMF (n=4)
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.2±0.6	0.9±0.3	0.6±0.1	0.8±0.3	0.5±0.2	0.6±0.5
GRB2	growth factor receptor-bound protein 2	1.7		1.9±0.1	2.1		1.5
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	1.7±0.3	1.9±0.3	2.2±0.4	2±0.3	2±0.1	1.9±0.4
MAP2K1	mitogen-activated protein kinase kinase 1	0.5±0.3	1.1±0.3	1±0.2	0.7±0.2	0.6	0.2±0.5
MAPK1	mitogen-activated protein kinase 1		1.5±0.3	1.2±0.1	1.7	−1.9	0.8
MKNK2	MAP kinase interacting serine/threonine kinase 2	1.4±0.7	1.7±0.2	1.6±0.4	1.7±0.8	1.7±1.03	1.6±0.9
MYC	v-myc myelocytomatosis viral oncogene homolog	−1.9±0.3	−1.3±0.7	−1.6±0.5	−1.7±0.3	−2.3±0.2	−1.4±1.02

polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF)

** p<0.01

* p<0.05 MPN vs. Control

htz-heterozygosity, hom – homozygosity, no mut – no mutation

Table 3:

Hematologic parameters in MPNs according to *JAK2*V617F status.

JAK2 V617F	Age years	F/M	Le x10^9/l	Er x10^{12}/l	Tr x10^9/l	Hb g/l	Hc %	MCV fl	LDH IU/l	β2 Mmg/l	Fibrin g/l	PT %	PTT s	ESR mm/h
PV+	68±10	2/4	9.7±3.1	6.5±0.8	588±145	157±17	0.48±0.04	75±9	523±90	4.5±1.5	3.1±0.3	33±28	30±4	2.3±0.8
ET+	55±14	3/2	8.7±1.7	4.9±0.8*	861±199	148±12	0.4±0.04	88±7	578±246	1.9±0.3**	3.5±1	52±36	32±3	14±11
ET-	60±17	4/2	9.4±1.2	4.7±0.5**	887±88	139±13*	0.4±0.04*	87±6	473±61	1.9±0.3**	3.8±0.9	74±24	27±8	17±9.5*
PMF+	65±13	2/5	11±2.8	5.1±0.7*	700±192	146±17	0.42±0.07	85±10	499±95	3.1±0.9	3.4±0.2	50±33	28±8	10±5.7
PMF-	58±12	3/2	7.9±2.8	3.9±0.8$^{***, \#}$	675±250	120±15$^{**, \#}$	0.35±0.03**	92±10*	527±176	2.4±1.1*	3.6±0.4	55±35	27±8	15±13
Normal			3.6–10	3.9–5.7	150–450	120–175	35–50	80–96	105–333	1–2.4	1.,5–4	≥70	25–35	<20

F/M-female/male ratio, Le- leukocytes, Er erythrocytes, Tr-thrombocytes, Hb-hemoglobin, Hc-hematocrit, MCV-mean corpuscular volume, LDH-lactate dehydrogenase, β2M-β2 microglobulin, Fibrin-Fibrinogen, PT-prothrombin time, PTT-partial thromboplastin time, ESR-Erythrocyte sedimentation rate, PV-polycythemia vera, ET-essential thrombocythosis, PMF-primary myelofibrosis

* $p < 0.05$

** $p < 0.01$

*** $p < 0.01$ vs. PV

$^{\#}$ $p < 0.05$ vs. PMF+

Table 4:

Overview of IL-6 induction of proliferation related signaling pathways in MPN granulocytes.

IL-6		PV	ET		PMF	
	Inhibitors	JAK2+	JAK2−	JAK2+	JAK2−	JAK2+
STAT3	JAK1/2	↓	↓	↑		
	JAK2	↓	↓	↑		↑
AKT	JAK1/2	↑		↑	↓	↓
	JAK2	↓		↑	↓	↓
MAPK	JAK1/2		↑	↑↑	↑	↑
	JAK2			↑	↓	↓

Solid cells: IL-6 stimulation, empty cells: IL-6 inhibition of signaling pathways, Inhibitors ↑-increase or ↓-decrease IL-6 effect, JAK2+: JAK2V617F positive and JAK2-: JAK2V617F negative MPN