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Interferon α Targets JAK2V617F Positive Hematopoietic Progenitor Cells and Acts through the p38 MAP Kinase Pathway

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Abstract

Objective—Interferon α (IFN α) therapy leads to hematological remissions and a reduction of the JAK2V617F allele burden in patients with polycythemia vera (PV). In this study, the cellular target by which IFN α affects hematopoiesis in PV patients was evaluated.

Materials and Methods—CD34⁺ cells were isolated from normal bone marrow and the peripheral blood of patients with PV and were treated in vitro with each of the three commercially available forms of IFN α : IFN α 2b, pegylated IFN α 2a (Peg-IFN α 2a) and pegylated IFN α 2b (Peg-IFN α 2b).

Results—Each form of IFN α was equally potent in suppressing hematopoietic colony formation by normal CD34⁺ cells, but Peg-IFN α 2a and IFN α 2b were more effective than Peg-IFN α 2b in inhibiting BFU-E derived colony formation by PV CD34⁺ cells. In addition, exposure of PV CD34⁺ cells to equal doses of Peg-IFN α 2a and IFN α 2b resulted in a 38–40% reduction in the proportion of JAK2V617F-positive hematopoietic progenitor cells (HPC), while equivalent doses of Peg-IFN α 2b did not reduce the number of malignant HPC. Further studies explored the mechanism by which IFN α induced PV HPC growth inhibition. Treatment of Peg-IFN α 2a increased the rate of apoptosis of PV CD34⁺ cells and the phosphorylation/activation of p38 MAPK in PV CD34⁺ cells, while the p38-specific inhibitor SB203580 reversed the growth inhibition and apoptosis induced by Peg-IFN α 2a.

Conclusion—These data suggest that low doses of IFN α selectively and directly suppress PV JAK2V617F HPC and that these agents act through the p38 MAP kinase pathway.

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Conflict of interest disclosure

The authors declare no competing financial interests.

Keywords

Polycythemia vera; interferon alpha; progenitor cells; p38 MAPK

Introduction

Polycythemia vera (PV) is a Philadelphia chromosome negative chronic myeloproliferative neoplasm (MPN) which is almost uniformly characterized by a mutation in JAK2¹⁻⁵. JAK2V617F has been shown to play a pivotal role in the origins of the MPN and to be an important diagnostic tool¹⁻⁵. Interferon α (IFN α) has been used to treat patients with MPN for over 20 years and has been shown to be effective in controlling thrombocytosis and limiting excessive red cell production^{6, 7}. In addition, IFN α therapy can correct marrow morphological changes associated with MPN, induce cytogenetic remissions and lead to the transition from monoclonal to polyclonal hematopoiesis in selected patients with MPN, indicating that IFN α might actually be capable of selectively targeting HPC belonging to the malignant hematopoietic clone⁸⁻¹¹. The widespread use of these therapeutic agents has been limited by adverse events which not infrequently occur in MPN patients treated with IFN α ^{6, 10-16}. In addition, IFN α therapy requires subcutaneous injection on repeated occasions each week which is occasionally unacceptable to some patients. Recently, two pegylated (peg)- forms IFN α , Peg-IFN α 2a (Pegasys) and Peg-IFN α 2b (Peg-Intron) have been approved for the treatment of patients with hepatitis C¹⁷⁻¹⁸. Both of these preparations have been evaluated for their ability to treat patients with MPN due their more favorable pharmacokinetic profiles which permits their being administered once a week without a loss of clinical efficacy¹⁹⁻²³.

Peg-IFN α 2b has been reported to be useful in treating patients with ET and PV^{19, 22}. More recently, Kiladjian et al.^{20, 21} have reported a trial of 40 patients with PV treated with peg-IFN α 2a. All patients enjoyed a hematological response within the first year of treatment and 92% of patients were able to tolerate the drug for at least 12 months. Furthermore, 24% of the entire cohort achieved a complete molecular remission while 3 additional patients had JAK2V617F allele burdens at the lower limit of detection. These data indicate that Peg-IFN α 2a might be capable of reducing or eradicating the JAK2V617F clone in a substantial proportion of patients²¹.

The mechanism action by which IFN α affects hematopoiesis in PV patients and whether one preparation of IFN α is more effective in treating patients with MPN remains the subject of a great deal of speculation. In the present study, we studied the effects of the various preparations of clinically available IFN α on normal and MPN hematopoietic progenitor cells (HPC) in vitro, in order to clarify their effect and relative potency on both normal and malignant hematopoiesis. Although the engagement of STAT proteins by activated IFN receptor-JAK complexes plays a role in IFN α mediated responses, several reports have implicated the mitogen activated protein kinase (MAPK) signaling cascades in IFN- α mediated biological responses²⁴⁻²⁷. Among the various MAPK pathways engaged by IFN α , signals generated by the p38 MAPK have been reported to play a role in mediating the inhibitory effects of IFN α on normal and malignant hematopoiesis. In the current study, we

also examined the role of activation of p38 MAPK pathway on IFN α -mediated effects on PV HPC.

Material and Methods

Isolation and purification of CD34⁺ cells from the peripheral blood of PV patients and normal bone marrow

PB was obtained from 15 PV patients after informed consent, was obtained according to the guidelines established by the Institutional Review Board of the Mount Sinai School of Medicine, New York, NY. All patients met the World Health Organization diagnostic criteria for PV and were JAK2V617F positive with an allele burden ranging from 20% to 95%²⁸. Patients were being treated with phlebotomy and aspirin therapy at the time of study. Fresh normal human bone marrow samples were purchased from ALLCELLS (Emeryville, CA). The PB and marrow samples were layered onto Ficoll-Hypaque (1.077g/mL; Amersham Biosciences) and low-density mononuclear cells separated after centrifugation. The CD34⁺ cell population was isolated using a human CD34⁺ cell selection kit (StemCell Technologies) according to the manufacturer's instructions. The purity of the CD34⁺ cell population was analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), the purity of CD34⁺ cells used in all experiments was 85%.

Hematopoietic progenitor cell assays

CD34⁺ cells were assayed in semisolid media as described previously²⁹. Briefly, 5×10^2 CD34⁺ cells were plated per dish in duplicate cultures containing 1 mL IMDM with 1.1% methylcellulose and 20% FBS, to which 50 ng/mL of SCF, TPO, FLT-3 ligand, IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF) and 2 units/mL erythropoietin (EPO) were added. Various concentrations of IFN α 2b, Peg-IFN α 2b (Schering Corporation, Kenilworth, NJ) and Peg-IFN α 2a (Roche, Nutley, NJ) (0, 100, 200, 500, 1000 and 2000 Units/ml) were added to these cultures upon their initiation. Colonies were enumerated after 14 days of incubation as previously described and individual colonies were plucked and genotyped for JAK2V617F²⁹.

Nested allele-specific PCR for JAK2V617F-positive colonies

Genomic DNA (g DNA) was isolated from randomized plucked colonies using the Extract-N-Amp Blood PCR Kits (Sigma, St. Louis, MO). JAK2V617F was detected by using a nested allele-specific PCR as previously described³⁰. The final PCR products were analyzed on 2.0% agarose gels. The nested PCR product had a size of 453 bp. A 279-bp product indicated allele-specific JAK2V617F positive whereas a 229-bp product denoted allele-specific wild type product. Colonies were classified as homozygous for JAK2V617F if they contained only the 279-bp band, whereas heterozygous colonies were identified based on the presence of both the 279-bp and 229-bp bands.

Flow-cytometric analysis

After treatment, cells were collected and washed with PBS for staining with Annexin V (BD Biosciences, San Jose, CA), phospho-p38 and cleaved caspase 3 (cell Signaling Technology, Danvers, MA), the staining procedures were performed according to the

protocols provided by the manufacturer. Data were acquired on a FACS Calibur flow cytometer (Becton Dickinson), and at least 10,000 live cells were acquired for each analysis (Cell-Quest software; Becton Dickinson).

Western blot analysis

Isolated PV CD34⁺ cells were treated with either Peg-IFN α 2a alone or treated initially with SB203580 for 30 min and then with Peg-IFN α 2a. Cells were harvested at designated time points. The protein extracts were prepared with RIPA lysis buffer (Boston BioProducts, Worcester, MA) and equivalent protein from each sample was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Phospho-p38 MAP kinase and p38 MAP kinase protein were visualized using the antibodies (Cell signaling, Danvers, MA) and ECL Western blotting reagents (Amersham LifeScience, Arlington Heights, IL).

Statistical analysis

Results were reported as the mean $\pm$ SD of individual data points obtained from varying numbers of experiments. Statistical significance was determined using Student's *t* tests or paired-samples *t* test. All *P* values are two-sided.

Results

Effects of different forms of IFN α on hematopoietic progenitor colony formation by normal bone marrow and PV peripheral blood CD34⁺ cells

In Figure 1A and B, the effects of increasing concentrations of IFN α 2b, Peg-IFN α 2b and Peg-IFN α 2a on the ability of normal human marrow CD34⁺ cells to generate CFU-GM and BFU-E derived colonies are shown. Each preparation of IFN α was equivalent in its ability to suppress CFU-GM and BFU-E derived colony formation by normal human marrow CD34⁺ cells. The half maximal inhibitory concentration (IC₅₀) of each of the three forms of IFN α as showed by their ability to inhibit CFU-GM and BFU-E derived colony formation was 2000 units/ml. The effect of each of these preparations of IFN α on peripheral blood CD34⁺ cells from PV patients is shown in Figure 1 C and D. IFN α 2b and Peg-IFN α 2a were each capable of suppressing BFU-E and CFU-GM derived colony formation by PV CD34⁺ cells to a greater extent than Peg-IFN α 2b. For instance, at concentration of 2000 units/ml, IFN α 2b and Peg-IFN α 2a diminished PV CFU-GM and BFU-E derived colony formation by 80 % and 90%, respectively. The IC₅₀ of both IFN α 2b and Peg-IFN α 2a was 200 units/ml for CFU-GM and less than 100 units/ml for BFU-E, while the IC₅₀ of Peg-IFN α 2b exceeded 2000 units/ml for PV CFU-GM and was 500 units/ml for PV BFU-E.

Effect of IFN α on eliminating the numbers of JAK2V617F positive hematopoietic colonies by PV samples

We next examined the ability of equal doses of each form of IFN α to reduce the numbers of JAK2V617F positive hematopoietic colonies assayed from PV CD34⁺ cells. The action of the 3 preparations of IFN α was compared by studying in parallel their effects on hematopoietic cell colony formation by CD34⁺ cells isolated from 6 different PV patients. Individual CFU-GM and BFU-E colonies were randomly plucked and the JAK2 genotype

determined using allele specific nested PCR. As can be seen in Figure 2A, 200 and 500 units/ml of IFN α 2b and Peg-IFN α 2a were each capable of reducing the numbers of JAK2V617F positive hematopoietic colonies. At a concentration of 500 units/ml of either IFN α 2b or Peg-IFN α 2a, the proportion of JAK2V617F-positive HPC was reduced by 38–40%, ($p < 0.05$). By contrast, the same dose of Peg-IFN α 2b did not alter the number of JAK2V617F positive hematopoietic colonies assayed (Figure 2 A) ($p > 0.1$). Both CFU-GM and BFU-E colonies that were assayed in the presence of each of the three forms of IFN α were composed of far fewer numbers of cells than those generated in the absence of IFN α (Figure 2B). In order to further identify the cellular target of Peg-IFN α 2a on JAK2V617F HPC, we performed similar studies on CD34 $^{+}$ cells from 9 additional PV patients for a total of 15 patients. The numbers of JAK2V617F positive hematopoietic colonies were reduced in 13 of 15 these cases treated with Peg-IFN α 2a in vitro (Table 1). Furthermore, in 11 of the 12 cases where JAK2V617F homozygous colonies were observed the addition of Peg-IFN α 2a led to either the elimination (5 of 11 cases) of such homozygous colonies or a marked reduction in their numbers (6 of 11 cases). In each of the 13 instances where IFN α reduced the number of colonies containing JAK2 mutated cells there was a concomitant increase in the numbers of colonies which contained exclusively WT JAK2 (Table 1). Remarkably, Peg-IFN α 2a treatment was far more frequently effective at decreasing the number of JAK2V617F homozygous HPC (91.7% of cases) than JAK2V617F heterozygous (53% of cases) HPC.

The inhibitory activity of Peg-IFN α 2a against PV CD34 $^{+}$ cells is associated with activation of p38 MAPK

The mechanism by which Peg-IFN α inhibited hematopoietic colony formation by PV CD34 $^{+}$ cells was further explored. As can be seen in Fig 3A, three days of treatment with 500 units/ml of Peg-IFN α 2a, significantly increased the numbers of PV CD34 $^{+}$ annexin V positive cells as compared to normal CD34 $^{+}$ cells. We next demonstrated flow cytometrically that the increased degree of the apoptosis was accompanied by increased numbers of CD34 $^{+}$ cells with a cleaved form of caspase 3, a mediator of apoptosis (Fig. 3B). The inhibitory effects of Peg-IFN α 2a on malignant HPC has been previously attributed to activation of the p38 MAPK^{24, 27}. As can be seen in figure 3B, addition of the p38 MAPK inhibitor SB203580 to Peg-IFN α 2a reduced the number of CD34 $^{+}$ cells with cleaved caspase 3 induced by Peg-IFN α 2a treatment alone. We further documented that the inhibitory action of Peg-IFN α 2a on PV CD34 $^{+}$ cells was related to phosphorylation of p38 MAPK by assessing the percentage of CD34 $^{+}$ cells with phosphorylated p38 flow cytometrically. Addition of Peg-IFN α 2a to PV CD34 $^{+}$ cells increased the percentage of CD34 $^{+}$ cells with phospho-p38, while the addition of phospho-p38 MAPK inhibitor, SB203580 alone had no effect on the number of CD34 $^{+}$ cells with phospho-p38. By contrast, the increased percentage of CD34 $^{+}$ cells with phospho-p38 was not observed when CD34 $^{+}$ cells were preincubated with SB203580 prior to treatment with Peg-IFN α 2a. The effect of Peg-IFN α 2a on the phosphorylation of p38 MAPK in PV CD34 $^{+}$ cells was further explored using western blotting (Fig. 3D), Peg-IFN α 2a was shown to induce the phosphorylation of p38 MAPK in PV CD34 $^{+}$ cells, an effect which was decreased by prior treatment with SB203580 followed by Peg-IFN α 2a. Furthermore, Peg-IFN α 2a treatment increased the level of phospho-STAT1 in PV CD34 $^{+}$ cells which, however, was not affected

by prior treatment with SB203580 (data not shown). To confirm the functional relevance of p38 MAPK activation following the exposure of the PV CD34⁺ cells to Peg-IFN α 2a, PV CD34⁺ cells were pre-incubated with the p38 MAPK inhibitor SB203580 and subsequently treated with Peg-IFN α 2a, and assayed for CFU-GM and BFU-E. Peg-IFN α 2a decreased the number of assayable CFU-GM and BFU-E derived colonies while the addition of SB203580 eliminated the inhibitory effects of Peg-IFN α 2a against both PV CFU-GM and BFU-E derived colony formation (Fig. 3E and F).

Discussion

IFNs are widely expressed cytokines that have potent antiviral and growth inhibitory activities^{31–33}. All type I IFNs bind to a common cell surface receptor, the type I IFN receptor which leads to activation of multiple signaling pathway^{31–33}. IFNs have a wide range of biological activities including increasing the cytotoxic activity of immune cells, increasing the expression of tumor associated surface antigens and major histocompatibility antigens, activating pro-apoptotic genes and proteins and modulating cellular differentiation^{7, 33}. For almost 25 years, IFN α has been recognized as a potent suppressor of hematopoiesis in vitro^{34, 35}. Furthermore, the administration of IFN α to patients with hepatitis C or a variety of malignancies is frequently accompanied by the development of cytopenias³⁶. Recently, however, IFN α has been shown in a murine system to also be capable of promoting cycling of quiescent hematopoietic stem cells, indicating that IFN α might differentially affect cells belonging to different stages along the hematopoietic cellular hierarchy^{37, 38}.

The relative effectiveness of the different preparations of IFN α that we have observed in vitro appears to have some correlations with the clinical activity of these drugs observed in MPN patients. Kiladjian and coworkers^{20, 21} have reported in a phase II trial that administration of Peg-IFN α 2a resulted in a reduction of the median granulocyte JAK2V617F allele burden in 40 patients from 45% to 22.5% after 12 months of treatment. Furthermore, the median JAK2V617F allele burden progressively decreased with longer periods of treatment being 17.5% at 18 months, 5% at 24 months, 5% at 30 months and 3% at 36 months. This same degree of reduction in the JAK2V617F allele burden was not observed by Samuelsson and coworkers⁴¹ following 24 months of Peg-IFN α 2b treatment in 25 patients with PV and ET. Two patients, however, have been reported by Larsen and coworkers⁴², who were retrospectively shown to have a profound molecular response following 36 months of Peg-IFN α 2b. Silver and coworkers have, however, reported that IFN α 2b therapy infrequently leads to molecular responses in PV patients and that a reduction in the JAK2V617F allele burden is not required to achieve a hematological response⁴³. These observed differences in clinical and molecular response rates with the various forms of IFN α therapy are likely due to a number of factors including the sensitivity of the malignant HPC to a particular form of IFN α , the doses of a particular form of IFN α that an individual patient can tolerate as well as the pharmacodynamic and pharmacokinetic characteristics of the form of IFN α used. Although we have shown that IFN α 2b and Peg-IFN α 2a in vitro appear to be more effective in eliminating malignant HPC, it remains unknown what number of malignant HPC must be eliminated in vivo to result in a reduction in a patient's granulocyte JAK2V617F allele burden.

The forms of IFN α evaluated in this study have been shown to be active in the treatment of hepatitis C^{17, 18}. Pegylation is a well established method of modifying the pharmacological properties of IFNs causing significant improvements in pharmacokinetics which in turn leads to improved efficacy. The two forms of Peg-IFN α studied differ however in structure, and pharmacokinetic properties¹⁷⁻¹⁸. The pegylation process also, has been shown to result in decreased biological activity of the core IFN using in vitro assays. This alteration of specific activity has been attributed to alterations in the interactions with specific receptors of IFN with the pegylated forms of these molecules leading to differences in intracellular signaling responses⁴¹⁻⁴⁵. Peg-IFN α 2a has been reported to result in a more limited STAT nuclear translocation, lower levels of IFN response gene mRNA and lower antiviral activity in vitro³⁹. These alterations in biological properties associated with the different forms of IFN α based upon in vitro analyses, however do not necessarily translate into distinct patterns of clinical efficacy. In several small randomized trials comparing Peg-IFN α 2b and Peg-IFN α 2a have been shown to be equally effective in treating hepatitis C and to have a similar safety profiles¹⁷⁻¹⁸. Such findings are consistent with the observation made in this report which shows that each of these forms of IFN α are equally effective in vitro in suppressing normal hematopoiesis. Whether these three forms of IFN α are equally effectively in suppressing malignant hematopoiesis remains the subject of speculation. In this study, we observed that in vitro that treatment with the same doses of either IFN α 2b or Peg-IFN α 2a was more effective than similar doses of Peg-IFN α 2b in eliminating in vitro JAK2V617F HPC. Such observations might be due to the structure of these forms of IFN and their unique patterns of interaction with the IFN receptor. A randomized clinical trial comparing that the efficacy of these three agents in patients with PV would be required to determine their relative efficacy and toxicity in treating MPN patients.

The mechanism by which IFN α affects MPN hematopoiesis remains the subject of a great deal of investigation. Several groups have suggested that IFN α might directly affect malignant HPC^{6, 15-16, 20-22, 30}, while Xiong and coworkers⁴⁶⁻⁴⁷ have provided evidence that IFN α therapy might act in vivo by eliciting an immune response to testis antigens aberrantly expressed by PV HPC. In the present report, however, we demonstrated that IFN α 2b, Peg-IFN α 2b and Peg-IFN α 2a are each capable of inhibiting in vitro the generation of CFU-GM and BFU-E by both normal marrow and PV CD34⁺ cells. The action of these various forms of IFN α on purified CD34⁺ cells indicates that this inhibitory activity is directly against the malignant HPC rather than being mediated through an accessory cell population. In addition, low doses of both IFN α 2b and Peg-IFN α 2a appear to be more effective in inhibiting PV HPC in vitro than Peg-IFN α 2b and to selectively eliminate cells with JAK2V617F positive HPC with persistence of wild type JAK2 HPC. In the current study, we documented that JAK2V617F homozygous CD34⁺ cells were more sensitive to the treatment with Peg-IFN α 2a than JAK2V617F+ heterozygous HPC. The mechanism by which Peg-IFN α 2a might preferentially eliminate JAK2V617F+ homozygous HPC can at present only be a subject of speculation, but is possibly due to an as yet unknown biological characteristic unique to homozygous but not heterozygous JAK2V617F + HPC. The increased number of assayable HPC with WT JAK2 which appear following treatment with Peg-IFN α 2a is likely due to a pool of such HPC which persist after elimination of the JAK2V617F + HPC⁴⁸. These findings indicate that IFN α directly targets both malignant and

normal HPC in vitro but does not eliminate the possibility that the clinical responses achieved with Peg-IFN α 2a might occur in part to the immune modulatory effects of this biological response modifier.

Several reports have shown that the p38 MAPK pathway is involved in IFN α signaling in a variety of cell types, suggesting this pathway plays an important role in IFN α mediated suppression of hematopoiesis^{24–27}. Mayer and coworkers provided the initial evidence that IFN- α activates the p38 MAPK pathway in chronic myeloid leukemia (CML) HPC which indicated that this signaling cascade plays a critical role in the induction of the anti-leukemic activities of IFN α in BCR/ABL expressing cells⁴⁸. Similarly, we have demonstrated that the p38 MAP kinase is activated in PV HPC by treatment with Peg-IFN α 2a and that the p38 MAP kinase specific inhibitor, SB203580 reverses IFN α induced CD34⁺ cell apoptosis and growth inhibition. These data indicate that the p38 MAP kinase pathway plays a similar role in IFN α -induced cell activity in PV HPC as was previously observed in CML⁴⁹. This observation suggests that the activation of this p38MAPK pathway by IFN α is not dependent on either BCR/ABL or JAK2V617F but might be due to some other characteristic which is common to each of the MPN. Additional data to substantiate this claim is provided by our observation that CD34⁺ cells from patients with primary myelofibrosis are also similarly responsive to the inhibitory actions of Peg-IFN α 2a (data not shown). IFN α , therefore, appears to be a potentially useful agent for the treatment of MPN patients which acts at the level of the HPC. Whether effects of the in vitro treatment with a particular form of IFN α might serve as a possible means of predicting individual patient responses to IFN α therapy will require careful validation in the future.

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A.

B.

C.

D.

Figure 1. Comparison of the effects of increasing concentrations of IFNa 2b, Peg-IFNa 2b and Peg-IFNa 2a on hematopoietic colony formation by normal bone marrow and polycythemia vera peripheral blood CD34⁺ cells

Hematopoietic colony numbers were enumerated after 14 days of incubation. **A**, CFU-GM derived colony formation assayed from normal BM CD34⁺ cells in the presence of varying concentrations of the three preparations of IFNa; **B**, BFU-E derived colony formation assayed by normal BM CD34⁺ cells in the presence of varying concentrations of the three preparations of IFNa; **C** CFU-GM derived colony formation assayed from CD34⁺ cells from PV patients in the presence of varying concentrations of the three preparations of

IFN α ; **D**, BFU-E derived colony formation assayed from CD34⁺ cells from PV patients in the presence of varying concentrations of the three preparations of IFN α ;

A. **B.****Size of PV hematopoietic colonies.**

Figure 2. Effects of the addition of equal doses of the three forms of IFNα on the number of JAK2V617F positive hematopoietic colonies assayed in vitro from CD34⁺ cells from patients with PV

CD34⁺ cells were incubated with either 200U/ml or 500U/ml of IFNα for 14 days and the numbers of colonies were enumerated. Individual colonies were randomly plucked and assayed for JAK2V617F. **A**, Comparison of the effects of the three forms of IFNα on JAK2V617F positive hematopoietic colony formation; (*: P<0.05; **: P<0.01; ***: p<0.001). **B**, Effects of addition of the three forms of IFNα at 500unit/ml on hematopoietic

colony size, in each panel the upper colonies are representative CFU-GM derived colonies and the lower colonies are representative BFU-E derived colonies.

Figure 3. Activation of the p38 MAPK pathway by Peg-IFNα 2a in PV CD34⁺ cells

A. Three days of treatment with Peg-IFNα 2a at a dose of 500units/ml significantly increased the number of apoptotic PV CD34⁺ cells as determined by flow cytometric analysis. **B.** Treatment with 500units/ml of Peg-IFNα 2a elevated the number of CD34⁺ cells with cleaved caspase 3 as determined by flow cytometrically. The elevation of CD34⁺ cells with cleaved caspase 3 was reduced by prior-incubation with SB203580 for 30 min at dose of 10 uM. **C.** FACS analysis also showed that the number of PV CD34⁺ cells with phosphorylated form of p38 were increased after treatment with 500units/ml of Peg-IFNα

2a, this increase was not observed with prior-incubation with SB203580 followed by Peg-IFN α 2a treatment. **D.** Western blotting demonstrated that the phospho-p38 level in PV CD34⁺ cells from two individual patients (PV1 and PV2) was increased after treatment with Peg-IFN α 2a, but the effect was not observed with prior-treatment with SB203580. Similarly, Peg-IFN α 2a inhibited both CFU-GM (E.) and BFU-E (F.) derived colony formation by PV CD34⁺ cells, this inhibitory activity was blocked by prior-treatment with SB203580. The colonies were enumerated after 14 days of incubation. (*: $p < 0.05$; **: $p < 0.01$).

Table 1
Effect of Peg-IFN α 2a Treatment on the JAK2 Genotype of PV Hematopoietic Colonies

Patients	Granulocyte JAK2 V617F allele burden (%)	JAK2 Genotype of Hematopoietic Colonies*						
		None				Peg IFN α 2a 500 U/ml***		
		Homo %	Hetero (%)	WT (%)	Total**	Homo %	Hetero (%)	WT (%)
PV 1	67	16.7	33.3	50	24	0	6.3	93.7
PV 2	45	8.3	66.7	25	24	0	50	50
PV 3	85	41.7	58.3	0	24	15.4	46.2	38.5
PV 4	>95	41.7	58.3	0	24	0	100	0
PV 5	28	16.7	66.7	17.6	30	31.6	26.3	42.1
PV 6	77	58.3	37.5	4.2	24	5	45	50
PV 7	60	42.9	50	7.2	14	0	47.6	52.4
PV 8	20	25	50	25	12	16.7	33.3	50
PV 9	70	36.4	36.4	27.5	11	0	50	50
PV 10	56	0	63.6	36.4	22	0	70.8	29.2
PV 11	90	0	92.8	7.2	28	0	55.6	44.4
PV 12	50	0	82.6	17.4	46	0	58.3	41.7
PV 13	90	100	0	0	20	50	0	50
PV 14	65	25	41.7	33.3	24	9.1	36.6	54.3
PV 15	95	90.9	9.1	0	22	70.8	25	4.2

Note: Homo: JAK2V617F Homozygous; Hetero: JAK2V617F Heterozygous; WT: contains only wild type JAK2;

* : Numbers indicate the percentage of total colonies with a particular JAK2 genotype for each patient studied.

** : The numbers indicate the total number of hematopoietic colonies that were genotyped; all cultures contained 500 CD34⁺ cells and individual hematopoietic colonies were plucked and analyzed.

*** : All cultures were treated with 500 units of Peg-IFN α 2a except PV7, in this case a response was observed with the addition of 200 Units of Peg-IFN α 2a., while no hematopoietic colonies formed in the presence of 500 units of Peg-IFN α 2a.