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Pegylated interferon Alfa-2a and hydroxyurea in polycythemia vera and essential thrombocythemia: differential cellular and molecular responses

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Polycythemia vera (PV) and essential thrombocythemia (ET) are chronic myeloproliferative neoplasms (MPNs) with high risk of thromboembolism and tendency to transform to myelofibrosis and acute leukemia. Pegylated interferon-α (PegINFa) is an effective treatment and unlike hydroxyurea (HU), it is reported to improve cytogenetic abnormalities, decrease *JAK2*V617F and *CALR* allelic burdens and achieve molecular remission [1–3]. We reported a return to polyclonal hematopoiesis in some females after PegINFa therapy [3], and unlike HU, PegINFa promoted proliferation and differentiation of PV and ET hematopoietic stem cells in vivo [4]. Cytokines, such as TNFα and interleukins are found to be elevated in MPN [5, 6] and are implicated as drivers of clonal expansion of *JAK2*V617F bearing cells [7]. Genome wide association studies have shown *IL28* SNP and treatment with PegINFa were associated with sustained virologic response in hepatitis C [8] and suggested favorable hematological response in 20 PV/ET patients [9].

Given these observations, we analyzed the effect of PegINFa and HU treatment on molecular response, clonal hematopoiesis (females), *TNFα* transcript levels, and *IL28B* haplotype as correlated with PegINFa response. We included 82 patients diagnosed with high-risk PV ($n = 45$) and ET ($n = 37$) (per WHO 2008) treated from 2008 to March 2017. Mutational analysis for *JAK2*V617F and *cMPL* was performed by quantitative allele-specific-PCR [10], and *CALR* mutations by semi-quantitative fragment analysis [11]. In females, SNPs of 5 X-chromosome genes (*G6PD*, *MPP1*, *FHL1*, *BTK* and *IDS*) were

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Author contributions

JP conceived and designed this study; TT collected and analyzed the data and drafted the manuscript; SS conceived TNFα studies and with SJK and JS performed the molecular analyses; KK isolated $CD34^+$ cells from the marrows, MS and NH analyzed cytokines, KH and SL secured IRB compliance. All authors approved the manuscript.

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genotyped and in heterozygotes transcriptional allelic frequencies of granulocytes were determined by quantitative allele-specific PCR. TaqMan Gene Expression Assays were used to determine *TNFα* and cytokine mRNAs and plasma levels of cytokines were measured. Relative *TNFα* expressions and *JAK2*V617F allelic burden were compared using paired t-test in GraphPad Prism (La Jolla, CA). As many of these patients participated in the MPD-RC trials the hematologic and clinical responses of these patients will be reported by the MPD-RC group.

*JAK2*V617F mutation was detectable in 70 patients, *CALR* mutation in 11 ET, *cMPL* G1544T in one ET (Supplementary Table 1). In total 31 patients (27 PV and 4 ET) in the PegINFa group, and 19 patients (17 PV and 2 ET) in the HU group had *JAK2*V617F mutant allelic burden >10% at baseline and with at least one 6-month follow-up allelic burden value (Supplementary Figure 1). Overall molecular response rate was 14 (45%) in PegINFa group and 17(77.2%) in the HU group. No complete molecular response rates were noted during the entire follow-up. Among 48 PegINFa treated patients, 29 had prior HU, and in this subset of HU refractory/intolerant patients, only 3 (16.6%) had molecular response. In all patients whose *JAK2*V617F allelic burden decreased to <5%, the allelic burden was retested using quantitative digital PCR, and their low detectable *JAK2* mutant burden was confirmed (range 0.42–4.3%).

In 20 patients, *JAK2*V617F allele burden was measured simultaneously in marrow $CD34^+$ and peripheral blood granulocytes, and the allelic burden in $CD34^+$ was lower than in granulocytes (Supplementary Figure 2), consistent with previous reports [4, 12].

We were able to determine clonality in 26 females (18 PV and 8 ET; 20 on PegINF and 6 on HU), and all were clonal prior to treatment. At the end of follow-up, all 6 patients on HU continued to be clonal but 2 (10%) patients on PegINFa converted to polyclonal. Their median time of HU and PegINFa treatment was 12 months (1–70) and 30 months (5–75). IL28 SNP *rs12979860* was determined in 44 patients who received PegINFa, and we found no correlation with clinical response (RR 0.86 (95%CI0.65–1.25)).

Pro-inflammatory cytokines suppress normal hematopoiesis and are elevated in MPN [5, 6]. In our initial pilot analyses, we noted increased TNFα by immunostaining in PV marrow with positive signal preferentially localized in immature cells and megakaryocytes (Supplementary Figure 3). We isolated cells of different degrees of differentiation from PV marrow and analyzed their *TNFα* transcript. Transcripts were higher in erythroid progenitors and the highest in $CD34^+$ stem cells, suggesting a possible role of TNFα on suppression of PV/ET clones (Fig. 1a). We then determined *TNFα* transcripts in CD34 + cells on 25 patients on whom samples were available before and after treatment (PegINFa=16 and HU=9), and peripheral granulocytes on 21 patients (PegINFa=12 and HU=9). After PegINFa therapy, there was a significant decrease in *TNFα* expression in $CD34^+$ cells ($p = 0.0004$), which paralleled the decline in *JAK2*V617F allele burden (Fig. 1b). The decline of both *JAK2*V617F allele and *TNFα* closely corresponded to PegINFa therapy, as shown in a patient in whom PegINFa had to be discontinued and switched to HU (Supplementary Figure 4). In HU-treated patients, there was no such decrease in either *TNFα* expression or *JAK2*V617F allelic burden (Fig. 1b). In contrast, TNFα expression in

peripheral granulocytes did not decrease after either therapy and did not correlate with the *JAK2*V617F allelic burden. We also measured transcripts of inflammatory cytokines *IL6*, *IL8*, *IL1B*, and *IL12A* in available residual RNA. Peripheral granulocytes of PV and ET patients had higher expression of these inflammatory markers compared to controls, but did not change after PegINFa therapy in marrow $CD34^+$ cells or granulocytes. However, after HU therapy, there was increased expression of *IL8* both in marrow $CD34^+$ cells and peripheral granulocytes, as well as increased *IL12A* in peripheral granulocytes (Supplementary Figure 5a–b–c). Concomitant plasma samples were available in 7 patients, which showed decreasing trend after PegINFa therapy ($p = 0.056$), while plasma IL6 levels ($p = 0.233$) and IL1B levels were unchanged (Supplementary Figure 5d). Thus, while other inflammatory markers are over-expressed in PV and ET, only *TNFα* shows decrease in response to PegINFa therapy.

To test whether PegINFa may directly regulate TNFα in MPN cells, we cultured PV erythroid progenitors in a liquid culture system that is optimized to support the proliferation of erythroid progenitors [13, 14]. PegINFa decreased *TNFα* mRNA in liquid culture, mimicking the pattern seen in patients (Fig. 2). To assess the causality of TNFα on suppression of PV clone, we used TNFα inhibitor, adalimumab, in semisolid (BFU-E) and liquid erythroid cultures in 5 PV patient samples (Supplementary Figure 6a). There was initial increase of *JAK2*V617F allelic burden until day 7 corresponding to erythroid expansion in the culture. However, following day 11 progressive decline of *JAK2*V617F allelic burden is noted due to the expansion of dormant non-PV erythroid progenitors. The *JAK2*V617F allelic burden decreased with adalimumab by 69% on day 11 ($p = 0.0047$) and 49% on day 14 ($p = 0.0235$). Thus, the addition of adalimumab favored the outgrowth of *JAK2* wild-type over *JAK2*V617F mutant alleles in erythroid progenitors as seen in BFU-E colonies (Supplementary Figure 6b), without any change in colony type or number of BFU-E colonies.

Although PegINFa treated patients had decreased $CD34^+$ cell *TNFα* transcripts which paralleled decreased *JAK2*V617F allelic burden, the plasma TNFα levels did not decrease to the same extent (Supplementary Figure 7). This quantitative discrepancy between *TNFα* mRNA expression level and its circulating protein after PegINFa therapy may suggest a paracrine inhibitory effect of TNFα. Although limited by the small number of samples, our data suggest that PegINFa suppresses *TNFα* expression, leading to a decrease in the *JAK2* mutant clone. We did not see any change in expression of other cytokines after treatment with either PegINFa or HU, suggesting only *TNFα* responded to PegINFa therapy. TNFα has been shown to induce apoptosis and necroptosis in hematopoietic stem cells [15]. Thus, it is tempting to speculate that TNFα-expressing *JAK2*V617F clones suppress surrounding normal stem cell cells, and PegINFa abrogates this suppression, thereby permitting normal polyclonal hematopoiesis to resume.

In summary, although both PegINFa and HU decreased *JAK2*V617F allele burden, no complete molecular remission was achieved. However, PegINFa may restore polyclonal hematopoiesis through abrogation of *TNFα* expression in the bone marrow progenitor cells, suggesting possible efficacy of combination of TNF inhibitor and PegINFa in elimination of the MPN clone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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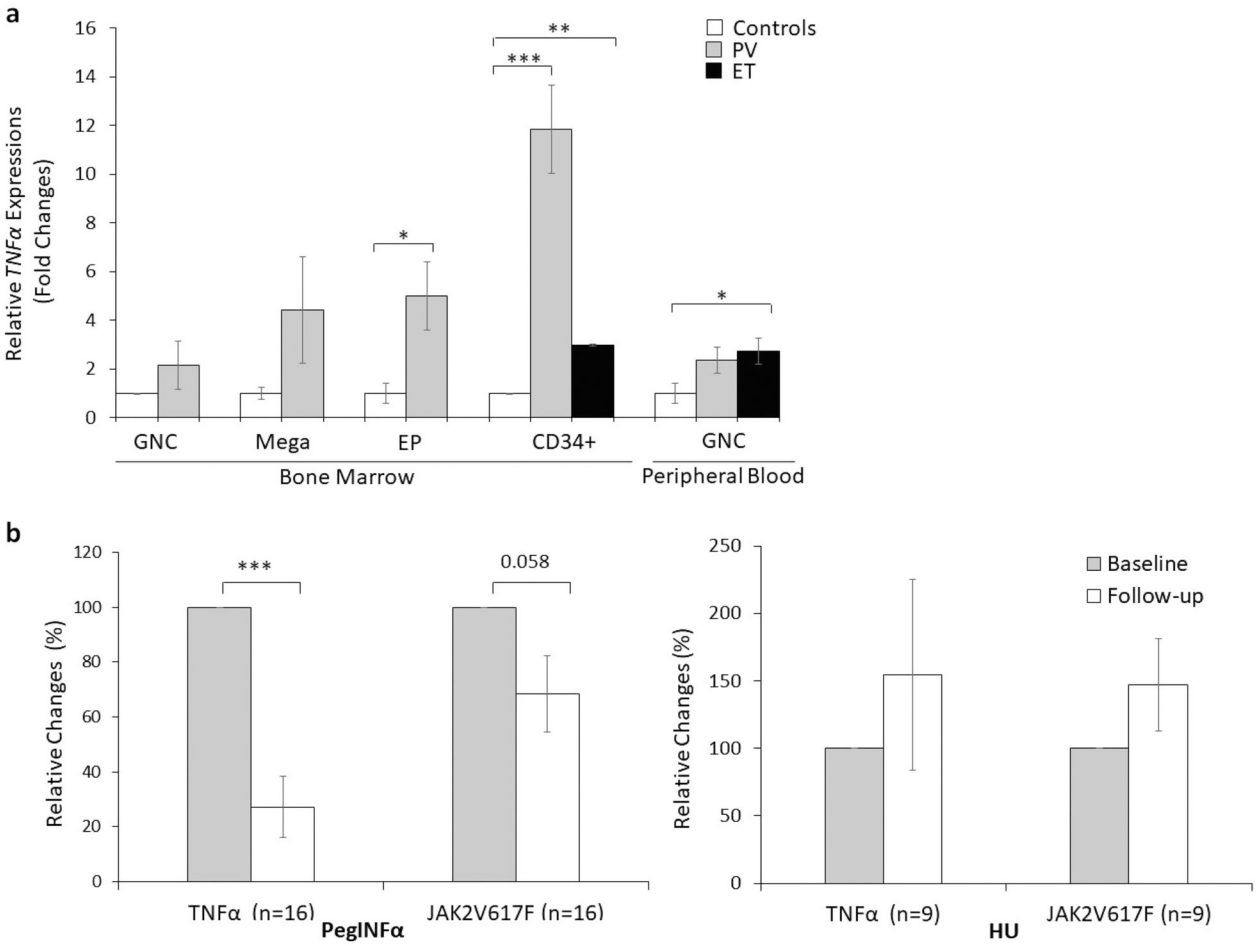

Fig. 1.

Relative *TNFα* expressions and *JAK2*V617F allelic burden in CD34 + cells and peripheral blood granulocytes. **a** *TNFα* expressions in CD34 + cells ($n = 4$) and peripheral granulocytes ($n = 7$) were normalized against *HPRT* and *GUSB*, calculated by ΔΔCt method, and expressed as a proportion change from the average of controls taken as1. **b** *TNFα* expression levels and *JAK2*V617F allelic burdens before and after PegINFa ($n = 16$) and HU ($n = 9$) in marrow CD34+ cells. TNFα expression levels were normalized against *HPRT* and expressed as a relative fold change against baseline as 100%. Relative allelic burden of *JAK2*V617F was calculated against baseline as 100%. *p <0.05; **p < 0.01; ***p < 0.0001

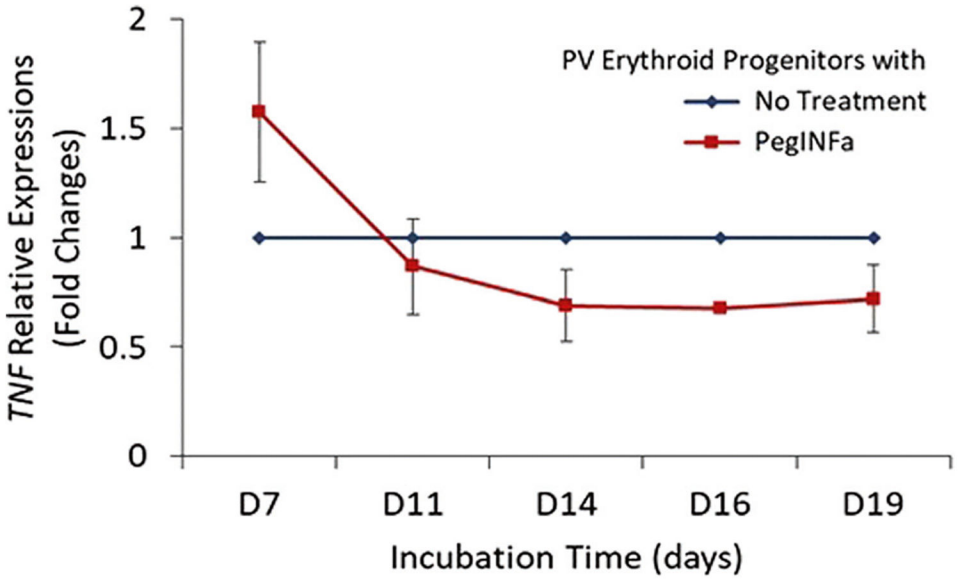

Fig. 2.

Effect of PegIFNa on *TNF* mRNA expression in erythroid progenitor cell in in vitro liquid cultures from PV patients ($n = 3$). Relative *TNFα* transcript with PegINFa was measured in erythroid progenitors and calculated compared to PV without PegIFNa as described in Fig. 1