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The *JAK2V617* mutation induces constitutive activation and agonist hypersensitivity in basophils of polycythemia vera

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ABSTRACT

Background

The *JAK2V617F* mutation has been associated with constitutive and enhanced activation of neutrophils, while no information is available concerning other leukocyte subtypes.

Design and Methods

We evaluated correlation of *JAK2V617F* mutation with the count of circulating basophils, the number of activated CD63$^+$ basophils, their response *in vitro* to agonists as well as the effects of a JAK2 inhibitor.

Results

We found that basophil count was increased in patients with *JAK2V617F* -positive myeloproliferative neoplasms, particularly in polycythemia vera (PV), and was correlated to the V617F burden. The burden of V617F allele was similar in PV neutrophils and basophils, while total JAK2 mRNA content was remarkably greater in the latter; however, the content of JAK2 protein in basophils was not increased. Higher number of CD63$^+$ basophils was found in PV patients compared to healthy subjects and patients with ET or PMF and was correlated to the V617F burden. Ultrastructurally, PV basophils contained an increased number of granules, most of which were empty suggesting cell degranulation *in vivo*. Ex-vivo experiments revealed that PV basophils were hypersensitive to the priming effect of IL-3 and to f-MLP-induced activation; pre-treatment with a JAK2 inhibitor reduced PV basophil activation. Finally, we found that the number of circulating CD63$^+$ basophils was significantly greater in patients suffering from aquagenic pruritus, who also showed higher V617F allele burden.

Conclusions

These data indicate that constitutively activated and hypersensitive basophils circulate at increased number in PV, underscoring a role of JAK2V617F in their abnormal function and, putatively, in pathogenesis of pruritus.

Key words: *JAK2V617F* mutation, basophil, polycythemia vera, pruritus.

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Introduction

The *JAK2V617F* mutated allele is present in virtually all patients with PV and in about 60% of those with essential thrombocythemia (ET) and primary myelofibrosis (PMF), that are the other two main clinical entities comprised within the group of myeloproliferative neoplasms (MPNs).[1] The presence of the mutation, and/or the burden of *JAK2V617F* allele, have been found to correlate with defined laboratory abnormalities and clinical features in the different MPNs.[2] In most of the studies performed in PV patients an allele burden greater than 50% was found to correlate with leukocytosis and higher hemoglobin level, lower platelet count, presence and degree of splenomegaly, occurrence of aquagenic pruritus and higher rate of transformation to myelofibrosis.[2]

JAK2V617F is a constitutively phosphorylated tyrosine kinase whose expression in cytokine-dependent cell lines conferred cytokine independence and cytokine hypersensitivity through the constitutive activation of STAT5, Akt and ERK-dependent pathways.[3,4] The adoptive transfer of marrow cells transduced with a retrovirus expressing *JAK2V617F* in irradiated recipient mice invariably resulted in the development of erythrocytosis,[5-9] eventually accompanied by leukocytosis, splenomegaly and later changes suggestive of myelofibrotic transformation.[6-9] Presence and burden of *JAK2V617F* correlated with endogenous erythroid colony formation in PV patients[10,11] and the expression of mutated JAK2 in mice induced Epo-independent growth *in vitro*.[7,9] Modification in the design of gene expression in murine models also resulted in an ET-like phenotype,[12,13] overall indicating that the *JAK2V617F* mutation is an integral component of the myeloproliferative process that underlies the different MPNs.

An unique gene expression profile has been associated with the presence and/or the burden of V617F allele in neutrophils; among involved genes there were some associated with neutrophil activation, such as PRV-1[14,17] and the gene encoding leukocyte alkaline phosphatas.[18] The constitutively activated status of circulating neutrophils associated with the mutated JAK2, together with enhanced activation of platelets and their hyperresponsiveness to agonists,[19,20] possibly contribute to the thrombotic tendency of PV.[21] On the other hand, information concerning functional relevance of the *JAK2V617F* mutation in other leukocyte subtypes, eosinophils and basophils, are not yet available. In this manuscript, we provide evidence that basophils circulate in an autonomously activated status at higher than normal level in PV patients, display ex-vivo hypersensitivity to agonists and that these abnormalities are corrected by treatment with a JAK2 inhibitor.

Design and Methods

Patients

This study involved a total of 78 PV patients in whom diagnosis satisfied the WHO criteria;[22] for comparison, we also included 70 patients with ET and 22 with PMF (all according to WHO criteria), and 7 subjects with reactive forms of hypoxic erythrocytosis. Most PV patients were under phlebotomy, but all patients were chemotherapy-free, at the time of blood sampling. Thirty-four healthy volunteers were included as controls. The study was approved by local Ethical Committee and an informed consent was obtained.

Flow cytometry analysis of activated basophils

Circulating $CD63^+/CD123^+$/HLA-DR- basophils were enumerated using 100 μL of eparin-anticoagulated peripheral blood (PB), promptly put in ice after sampling; antibodies were from Becton Dickinson (San Jose, CA, USA). At least 200,000 events were acquired on a FACScan flow cytometer; results were expressed both as the percentage of gated basophils expressing CD63 and as the absolute number of $CD63^+$ basophils by normalizing to total basophil count. CD63 expression level was calculated as the ratio of geometric mean fluorescence intensity (MFI) with isotype control antibody.

Purification of basophils and granulocytes

Basophils were purified from PB using a negative-depletion immunomagnetic procedure (Miltenyi Biotech; Gladbach, Germany). Purity of the isolated basophil preparations was checked by flow cytometry after labelling with PE-CD123/PerCP-HLA-DR monoclonal antibodies (Becton-Dickinson); median purity was 81%, ranging from 75 to 86%. Neutrophils were obtained after centrifugation of PB on a Ficoll density gradient; by visual inspection of cytosmears, neutrophils were greater than 95-97% of the cells while basophils were virtually absent from these cell suspensions.

Analysis involving DNA and RNA

Determination of the JAK2V617F burden in density-gradient purified neutrophils and immuno-selected basophils was performed using real-time PCR.[16] For discriminating between un-mutated and V617F-mutated JAK2 mRNA in purified neutrophils or basophils we employed an ARMS technique, as previously described.[16] Quantification of the level of total (mutated plus wild-type) JAK2 mRNA was accomplished with TaqMan® Gene Expression Assays (HS-00234567_m1; Applied Biosystems, Foster City, CA, USA) by means of ABI PRISM 7300 HT Sequence Detection System. Gene expression profiling was achieved using the Comparative cycle threshold (CT) method of relative quantitation using VIC-labeled RNaseP probe as the housekeeping gene (Applied Biosystems) (DCT).

Determination of JAK2 protein content in basophils and granulocytes

Neutrophils and basophils were purified from PB as described above; to obtain enough protein amount for western blotting analysis, basophils and neutrophils from three PV patients with greater than 50% V617F allele burden and three normal controls were pooled. Cell lysates were resolved on a 10% acrylamide SDS/PAGE and blotted onto a PVDF membrane

(Immun-Blot PVDF Membrane, BioRad, Hercules, CA). Blots were probed with antibodies specific for JAK2 (anti-Jak2 rabbit antibody, Cell Signaling Technology, Danvers, MA, USA) and tubulin (β-Tubulin mouse monoclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA), followed by peroxidase labelled secondary antibodies, and revealed with ECL (Amersham ECL Western Blotting Detection Reagent, Ge Healthcare, Little Chalfont, UK). To measure cellular JAK2 protein content we also employed a FACS-based technique. Samples of PB from PV patients and healthy controls were incubated with CD45 PerCP (Becton Dickinson, USA) and CD11c PE (BD Pharmingen, USA) for neutrophils or CD45 PerCP and CD203c PE for basophils at room temperature for 15 minutes in the dark. Samples were fixed by mixing one volume of blood with 20 volumes of pre-warmed 1X BD Phosflow Lyse/Fix Buffer (Becton Dickinson, USA) at 37°C for 10 minutes. After washing twice with BD PharmingenTM Stain Buffer (Becton Dickinson, USA), cells were permeabilized by adding 1 mL of BDTM Phosflow Perm Buffer III (Becton Dickinson, USA) followed by an anti-JAK2 rabbit antibody at room temperature for 30 minutes in the dark, washing, incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, USA), and finally resuspended in the same buffer prior to flow cytometric analysis using FacsCan (Becton Dickinson, USA). Data analysis was performed using WinMDI software (v2.9; *http://facs.scripps.edu/software.html*).

Ex vivo stimulation of basophils

PB samples were incubated with varying concentrations of recombinant hIL-3 and/or N-Formyl-Met-Leu-Phe (fMLP) peptide and the appearance of CD63 on the membrane of $CD123^+$/HLA-DR- gated basophils was measured. In some experiments, the specific JAK2 inhibitor AZD1480 (kindly provided by Astra Zeneca Ltd) was used. PB samples (100 μL volumes) collected in preservative-free heparin were processed immediately after sampling. Samples were equilibrated at 37°C in a water bath in polypropilene tubes for 15 min; then, rhIL-3 (from 0.1 to 10 ng/mL; Peprotech Inc, Rocky Hills, NJ) and fMLP peptide (from 0.01 to 0.04 μM; Sigma, Milano, I) were sequentially added, and an additional 15 min incubation was performed. Control tubes containing no addition (blank), rhIL-3 or fMLP alone (controls) were also prepared. At the end of incubation, samples were put on ice for 5 min, and basophils were labelled with 20 μL of FITC-CD63, PE-CD123 and PerCP anti-HLA-DR antibody cocktail (BD FastImmune, Becton-Dickinson) for 15 min at room temperature. Red blood cells were lysed with 2 mL of 1x FACSTM Lysing solution (Becton-Dickinson) for 15 min at room temperature and nucleated cells were washed twice with 1-2 ml of PBS.

Quantification of $CD63^+$ cells was performed in the basophil gate by acquiring at least 200,000 events; each experimental point was performed in duplicate. For inhibition of JAK2-mediated responses, cell samples were pre-incubated for 15 min at 37°C with the JAK2 inhibitor AZD1480 using two different concentrations (400 and 4,000 nM); then, optimal amount of rhIL-3 (10 ng/mL) and f-MLP peptide (0.04 mM) were added, and the cells were analyzed as described above.

Trasmission electron microscopy (TEM)

The enriched PB mononuclear cell fraction, obtained after centrifugation over a Ficoll-Hypaque gradient (Lymphoprep, Nycomed Pharma; Oslo, Norway), was processed for trasmission electron microscopy (TEM) by fixing in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 2 hr at 4°C and postfixing in osmium tetroxide for 60 min at 4°C. The samples were then dehydratated in alcohol at progressively higher concentrations and embedded in Spurr resin (Poliscience, Warrington, PA, USA). Consecutive thin and ultrathin sections were cut using a Reichert ultramicrotome. Ultrathin sections were collected on 200-mesh copper grids, counterstained with uranyl acetate and lead citrate, as described.[23] Both the total number of granules per cell, and the number of empty granules, were enumerated in at least 10 basophils/sample.

Statistical analysis

Comparison between groups was performed by the Mann-Whitney U or Fisher test as appropriate, using the SPSS software (StatSoft, Inc., Tulsa, OK, *http://www.statsoft.com*), GraphPad InStat software (GraphPad Software, Inc., San Diego, *http://www.graphpad.com*) or ORIGIN software (V 7.5, OriginLab Northampton, MA, USA, *http://www.originlab.com*) for computation. The analysis of correlation between JAK2V617F allele burden and hematologic parameters was performed according to Spearman's rank nonparametric correlation test. A P value of less than .05 was considered to indicate statistical significance; all tests were two-tailed.

Results

We studied a cohort of 78 *JAK2V617F*-mutated PV patients in whom the level of mutated allele burden ranged from 1 % to 100%, with a median value of 56%; their main hematological and clinical features are reported in Table 1. About half of the patients referred a history of aquagenic pruritus, that was considered by the referring physician as possibly related to the underlying hematologic disease after careful exclusion of any other known potential cause, and when it was described from the patient as diffuse, non-occasional, itching exacerbated by water contact and resistant to common anti-histamine drugs, whenever used. Due to the design and objectives of the study, the percentage of patients with aquagenic pruritus can result over-estimated and does not reflect the overall occurrence of this symptom in an unselected population of PV patients. We also evaluated 70 ET patients, 62% of whom were *JAK2V617F* mutated (mean allele burden was 29%) and 17 patients with PMF (13 of whom had the V617F allele, mean allele burden was 49%).

First, we found that mean absolute count of circulating basophils, measured by routine coulter counter, was significantly greater than control subjects (23.5±8.7 $x10^6$/L) in patients with PV (119.8±93.8$x10^6$/L;

Table 1. Clinical and hematological features of the patients with polycythemia vera (n=78) included in the study.

Demographic characteristic	
Median age, years (range)	62 (17-84)
Male sex, no. (%)	37 (47)
Laboratory and clinical features	
White cell count, $\times 10^6$/L	
Mean	11.1±4.8
Median (range)	9.6 (4.6-28.3)
Hemoglobin, gr/dL	
Mean	15.0±1.7
Median (range)	14.8 (13.3-18.9)
Hematocrit, %	
Mean	47.5±4.7
Median (range)	47.4 (42.9-57.5)
Platelet count, $\times 10^6$/L	
Mean	499.0±226.0
Median (range)	468.0 (132-1,101)
JAK2V617F mutated pts, no. (%)	78 (100)
1-50%	35 (45)
50-100%	43 (55)
Median *JAK2V617F* allele burden, % (range)	56 (1-100)
Pruritus, no. (%)	38 (49)
Arterial or venous thrombosis, no. (%)	24 (31)
Palpable splenomegaly, no. (%)	48 (62)

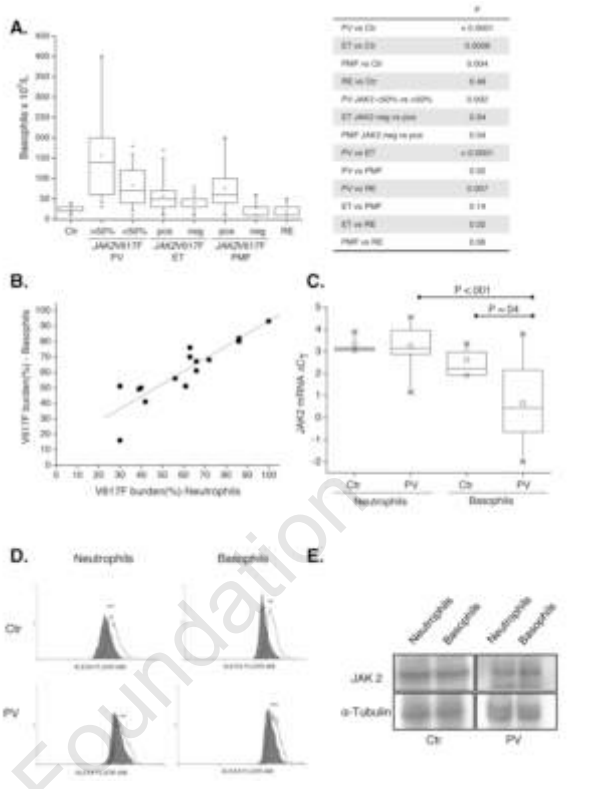

Figure 1. (Panel A) The absolute count of peripheral blood basophils in PV patients (n=78) divided according to their JAK2V617F allele burden lower or greater than 50%; patients with ET or PMF, control subjects (Ctr) or subjects with reactive forms of erythrocytosis (RE) are also shown. Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line in the box marks the median, the small square inside indicates the mean value, and bars show the range of values. The P value of the differences among different patient groups is shown on the right. (Panel B) Correlation between the burden of JAK2V617F allele concurrently measured in density gradient-purified neutrophils (on the X-axis) and immunomagnetically selected basophils (on the Y-axis) in PV patients (n=15). (Panel C) Level of total JAK2 mRNA in purified neutrophils and basophils from healthy control subjects and PV patients was determined with real time PCR and expressed as DCT after normalization to RNAseP as the housekeeping gene. Please note that higher DCT values indicate lower mRNA content. (Panel D) FACS analysis for intracellular JAK2 staining in neutrophils (on the left) and basophils (on the right) using whole peripheral blood samples; for details, refer to the text. Gray area represents unspecific fluorescence. The Y-axis indicates mean fluorescence intensity (MFI; arbitrary units). (Panel E) Western blot analysis of JAK2 content in neutrophils and basophils pooled from three patients with PV (all with >50% V617F allele) and three healthy subjects. Tubulin was used to normalize protein load.

$p<0.0001$), patients with ET (48.9±30.0×10^6/L; $p=0.0006$) or with PMF (64.1±59.3×10^6/L; $p=0.0042$); on the other hand, patients with reactive forms of erythrocytosis had basophil count similar to healthy subjects (20.0±15.3×10^6/L; $p=0.46$) (Figure 1A). The basophil count was significantly greater in patients with PV compared to ET or PMF patients ($p<0.0001$ and =0.02, respectively). There was also a statistically significant difference in mean basophil count between PV patients harboring a lower or greater than 50% V617F allele burden (82.8±48.9 and 155.5±109.7×10^6/L, respectively; ($p=0.002$) and between ET or PMF patients who were V617F mutated or wild-type (55.9±35.8 and 39.6±19.0×10^6/L , respectively; $p=0.04$ in case of ET; 75.4±63.0 and 24.3±18.1×10^6/L, respectively; $p=0.04$ in case of PMF) (Figure 1A).

We measured the burden of V617F allele in immunomagnetically selected basophils and in density-gradient purified neutrophils from 15 PV patients; we found that the two measurements were strongly correlated each to the other (r=0.90; Figure 1B), suggesting that the relative proportion of wild-type and mutated *JAK2V617* allele in the two leukocyte subtypes was comparable. On the other hand, we observed that the content of total JAK2 mRNA was significantly greater in PV basophils compared not only to normal basophils (P=.04) or neutrophils ($p=0.01$; n=12 subjects) but also to the concurrently purified PV neutrophils ($p=0.00007$) (Figure 1C). Such an increase was not due to a preferential transcription or increased stability of mutated JAK2 mRNA in PV basophils, since the relative proportion of the wild-type and V617F-mutated mRNA transcripts was consistent with the results of quantitative (*not shown in detail*). To evaluate whether also the content of JAK2 protein was increased in PV basophils, we employed a FACS-based analysis in three PV patients and three healthy controls, and western blotting technique; in the latter instance, we pooled purified basophils and granulocytes from three additional PV patients and three healthy controls to cope with the low protein recovered. However, using both approaches we were unable to document significantly increased JAK2 content in basophils (Figure 1D

and E). In particular, the mean fluorescence index (MFI) measured in PV basophils was 1,083±1,028, similar to 1,400±762 in control basophils, and 341±186 and 540±339, respectively, in PV and normal granulocytes. Therefore, we concluded that the increased JAK2 mRNA levels in PV basophils did not result in increased protein synthesis.

The expression of CD63 on the outer membrane of basophils was used as a marker of their activated status both *in vivo* and in *ex vivo* experiments.[24] We found that mean percentage of $CD63^+$ cells in the $CD123^+$/$HLA\text{-}DR^-$ basophil gate was significantly greater in PV patients than in controls (13.0±10.5% versus 1.8±1.7%; p=0.01), patients with ET or PMF, or patients with reactive erythrocytosis (Figure 2A). The absolute number of activated basophils in the circulation increased from 0.4±0.3×10^6/L in controls to 15.5±11.6×10^6/L in PV patients (Figure 2B; p=0.004); the number of $CD63^+$ basophils in ET or PMF was similar to controls (Figure 2B). Both the relative proportion and the absolute number of CD63+ basophils in the PB were correlated to the burden of V617F allele; mean values were 15±9.5% and 21.2±10.8×10^6/L, respectively, in PV patients with >50% mutated allele compared to 4.7±3.8% and 7.1±6.7×10^6/L in those with a mutated allele burden <50% (p=0.02, and p=0.04, respectively; Figure 2A, 2B). In addition, we found a significant linear regression between the absolute number of circulating $CD63^+$ basophils in PV and the JAK2V617F allele burden (r=0.73, P=.008; not shown in detail). Also the CD63 MFI was significantly increased in PV patients compared to control subjects (1,405±856 vs 740±145.2; P=.04) as well as to patients with ET or PMF or subjects with reactive erythrocytosis (Figure 2C); on the other hand, although the mean MFI value was greater in patients with more than 50% V617 allele (1,423±1,112) compared to those with <50% mutated allele (1,152±604), the difference did not reach the significance level because of the wide scattering of data (Figure 2C).

We employed TEM analysis to enumerate the number of granules contained in basophils and to characterize their morphology; a representative image of control and PV basophils is presented in Figure 3A. We found that the number of granules contained in basophils of PV patients was significantly greater than in healthy controls (18.1±1.4 versus 10.0±1.1, respectively; p=0.005); furthermore, the number of empty granules, mostly of which also presented a disrupted membrane, was significantly increased in PV compared to control basophils (7.4±0.9 versus 0.1±0.1, respectively; p<.001) (Figure 3B). In most instances, the cytoplasm of PV basophils also showed abnormal electrondensity and extensive vacuolization (Figure 3A).

Altogether, these data suggested that the number of activated basophils circulating in PV patients is increased over control subjects and correlated to the V617F allele burden; furthermore, these cells presented morphological abnormalities compatible with ongoing in-vivo activation and degranulation.

To evaluate possible correlations between the *JAK2V617F* mutation and basophil function, we evaluated their *ex vivo* activation by monitoring the expression of the activation marker CD63 on the cell surface; to this end, cells were incubated with rhIL-3, known to exert a potent priming effect mainly through the JAK2/STAT5 pathway,[25] followed by challenging with the f-MLP peptide, that acts by binding to a heterotrimeric G-protein coupled receptor. We found that at any of the three f-MLP concentrations employed (from 0.01 to 0.04 uM) the fraction of basophils induced to express CD63 was significantly greater in PV patients than in controls, particularly in those having greater than 50% mutated allele (Figure 4A). At the highest dose of 0.04 mM f-MLP employed, there were 2.44±0.6-fold more basophils expressing CD63 in PV compared

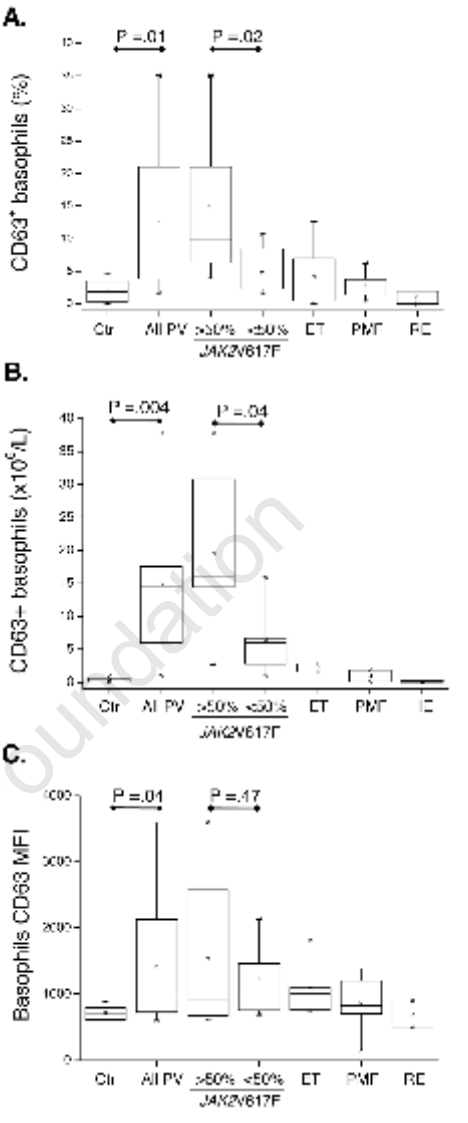

Figure 2. **The plots show the fraction of basophils expressing the CD63 activation marker within the basophil gate (Panel A) or the absolute count of $CD63^+$ basophils (Panel B) in the PB of PV patients, either all together (n=72) or divided according to their JAK2V617F allele burden lower or greater than 50%. Patients with ET or PMF, control subjects (Ctr) or subjects with RE are also shown. The mean fluorescence intensity of CD63 on the membrane of gated basophils is presented in (Panel C).**

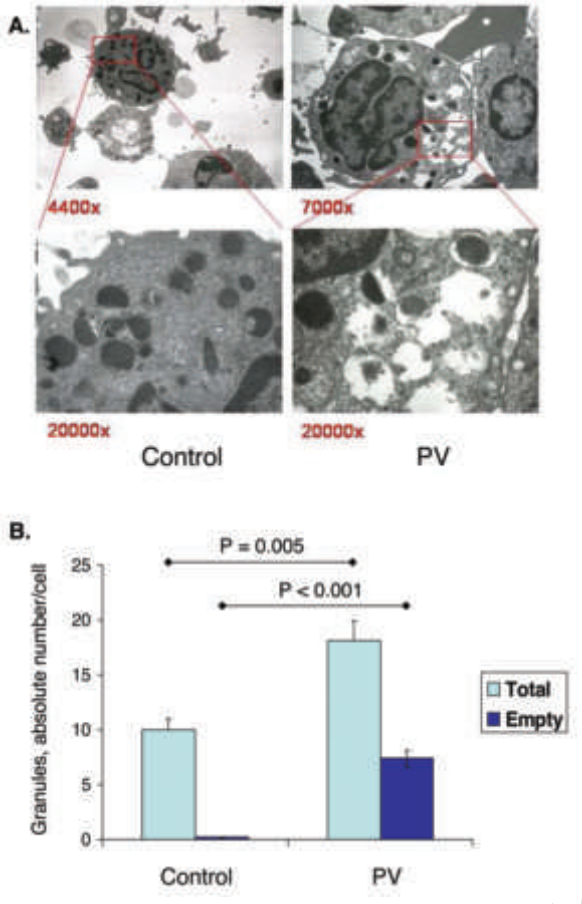

Figure 3. (Panel A) Representative TEM analysis of circulating basophils in a control subject (images on the left) and a PV patient (on the right; V617F allele burden =70%). Thin (upper panels) and ultrathin (lower panels) sections were observed under vacuum with an EM 109 Zeiss microscope equipped with built-in electromagnetic objective lenses and camera (Oberkochen, Germany). Photographs were taken with Kodak Technical Pan film (Kodak, Rochester, NY, USA), developed with Kodak D 19 1+4 automatic developer and scanned with an EPSON Perfection 3200 photoscanner (Seiko EPSON, Nagano-ken, Japan). Original magnification was 4,400x and 7,000x for the upper left and right panel, respectively, and 20,000x for the lower panels. (Panel B) The absolute number of granules contained in basophils from PV patients (n=5) and healthy subjects (n=4) is shown (gray columns) after enumerating at least 10 basophils/subject; the number of those granules devoid of their electron-dense content (empty granules) is also presented (black columns). Statistically significant differences are reported in the plot.

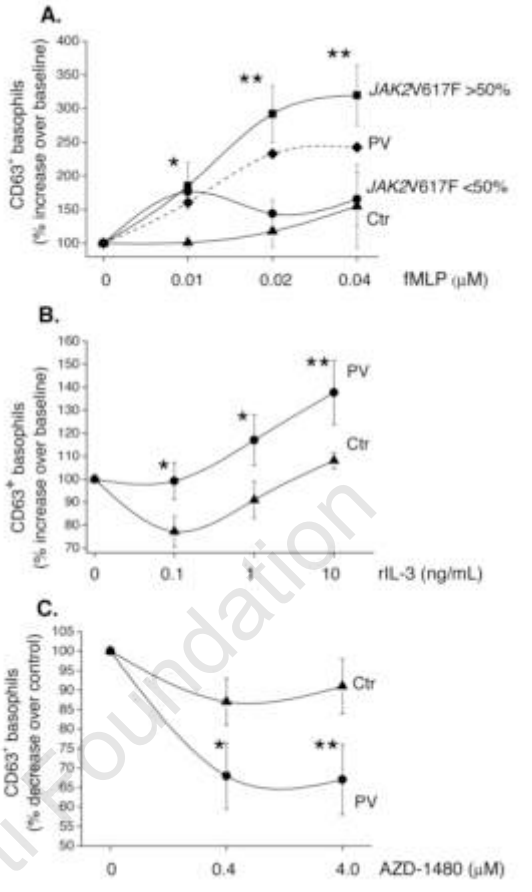

Figure 4. (Panel A) Expression of the activation marker CD63 in PB cells after being incubated ex-vivo with increasing amounts of fMLP peptide (0 to 0.04 mM) in the presence of an optimal amount of rhIL-3 (10 ng/mL). Results were expressed as per cent increase of CD63+ basophils over un-stimulated cells. The mean (±SD) values measured in control subjects (n=5; triangles) and PV patients (n=10), either all together (dashed line; for clarity, SD is not presented) or divided according to their V617F allele burden (>50% (squares) or <50% (dots), n=5 each), is presented. (Panel B) Experiments as above were performed by using increasing amounts of rhIL-3 in the presence of a fixed dose of f-MLP peptide (0.02mM). Only PV patients with >50% mutated V617F allele were used in these experiments and compared to controls (n= 5 each). Results were expressed as per cent increase of CD63+ basophils over cultures containing f-MLP only. (Panel C) PB cells from PV patients and control subjects (n= 5 each) were pre-incubated with the specific JAK2 inhibitor AZD1480 at two different concentrations, and then challenged with fMLP peptide (0.04 mM) and IL-3 (10 ng/mL). The fraction of cells in the basophil gate expressing CD63 was measured by FACS; results were expressed as per cent decrease of CD63+ basophils in wells containing the drug compared to cells without inhibitor. Only PV patients with >50% mutated V617F allele were used in these experiments. *p<0.05; **p<0.01.

to control samples (1.38±0.3 fold increase over baseline); in patients with >50% mutated allele the increase of CD63+ basophils compared to baseline was 3.3±0.2 fold (p<0.01; Figure 4A). Similarly, when basophils were primed with varying amounts of rhIL-3 (in the range from 0.1 to 10 ng/mL) and then challenged with an optimal amount of fMLP, the response of PV cells resulted significantly greater than in control cells at any IL-3 dose point (Figure 4B). Overall, these data suggested that the response of PV basophils to the priming effect of IL-3 is abnormally enhanced compared to control cells. To address the role of mutated JAK2, we employed the potent and selective JAK2 inhibitor AZD1480. This agent was shown able to significantly reduce the *ex vivo* activation of PV basophils in response to optimal amount of f-MLP and rhIL-3 (Figure 4C); at 4.0 mM AZD-1480, there was a 66% reduction in the fraction of PV basophils expressing CD63. Notably, the inhibitory effect was more pronounced in PV than control basophils, which were not appreciably affected by the drug. We found no meaningful correlation between number of circulating CD63+ basophils and hematological or clinical characteristics of PV patients, which

included splenomegaly, thrombosis, or need of chemotherapy (not shown in detail); on the other hand, we found that both the relative proportion and the absolute count of circulating $CD63^+$ basophils were significantly higher in patients suffering from aquagenic pruritus than in those who did not (Figure 5). Also, according to previous reports,[26,27] we found that the V617F allele burden was significantly greater in patients with pruritus (71±18%) than in those without (48±19%; p=0.002).

Discussion

To the best of our knowledge, this is the first study addressing possible effects of the *JAK2V617F* mutation in basophils from patients with PV and other MPNs. The data presented herein suggested that: (i) the count of basophils in the PB of MPN patients, but particularly in those with PV and in case of *JAK2V617F* mutated ET or PMF, is significantly increased over normal level. The design of this study does not allow to conclude whether this is actually due to an increased output from *JAK2V617F* mutated basophil progenitors, increased size of the early progenitor pool, increase survival of the mature cells, or a combination of these. At this regard, it is intriguing that IL-3 was recently shown to efficiently protect normal basophils from apoptosis through the activation of BCL-XL and a Pim-1 dependent pathway.[28] (ii) the count of constitutively activated basophils in the circulation, as measured by their expression of the activation marker CD63, is significantly increased in PV patients; intriguingly, their number is associated with the highest allele burden and with the complaint of aquagenic pruritus. Of note, patients with ET or PMF had count of activated basophils that did not differ significantly from healthy subjects. Indirect support to an in-vivo activated status of PV basophils was also provided by the findings of an increased number of empty granules in these cells according to electron microscopy analysis. (iii) *in vitro*, PV basophils showed hypersensitivity to IL-3 and were hyper-responsive to the f-MPL agonist compared to normal cells; (iv) abnormal *in vitro* activation was largely prevented by treatment with a JAK2 inhibitor. One additional findings of this study is that the content of total JAK2 mRNA in PV basophils was significant increased compared to PV granulocytes as well as to control basophils, without evidence of preferential transcription or accumulation of V617F mutated RNA. To ascertain whether also the content of JAK2 protein was increased in PV basophils, we performed FACS analysis and western blotting; results obtained with both techniques indicated that the protein content did not differ in PV basophils compared to PV granulocytes or normal cells. Due to the low number of basophils which could be recovered after immunomagnetic purification we have been unable to perform experiments aiming at distinguishing between increased JAK2 mRNA transcription from increased mRNA stability as the mechanism(s) for the higher levels of JAK2 mRNA measured in basophils. However, it is of interest that these findings are reminiscent of PRV-1 gene,

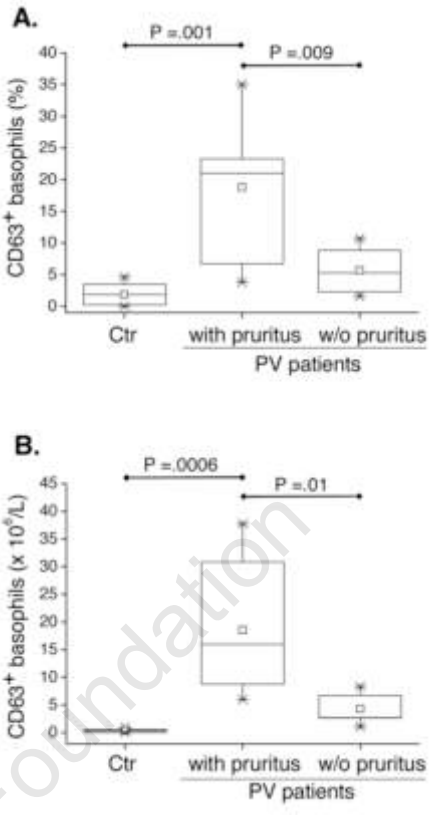

Figure 5. Plots show the percentage of basophils expressing the CD63 activation marker (Panel A) or their absolute count (Panel B) in the PB of PV patients according to the complaint or not of aquagenic pruritus.

whose expression was found enhanced in PV granulocytes without being associated with increased protein content.[29]

To evaluate the activation status of circulating basophils and their response in vitro to agonists we measured the expression of CD63 on the basophil cell membrane. CD63 is a tetraspanin contained in the inner granule surface in resting basophils; its expression on the outer cell surface correlates with basophil degranulation and histamine release, and serves as a reliable marker of allergen-induced basophil activation.[24] The effector functions of basophils are potently enhanced by several cytokines, including IL-5, GM-CSF, nerve growth factor; however, IL-3 is the most potent priming cytokine for human basophils, enhancing mediator secretion, the production of IL-4 and IL-3, the de-novo synthesis of leukotriene C4 and granzyme B.[25,30,31] IL-3, as well as IL-5, induces JAK2 and STAT5 phosphorylation as a non-redundant mechanism for basophil activation.[25] Data from *ex vivo* experiments meaningfully supported a hyper-sensitivity of PV basophils to functional activators possibly ascribable to constitutive signalling from mutated JAK2, as revealed by the priming effects of low-dose IL-3 and enhanced response to fMLP peptide, and by the inhibition produced by a potent JAK2 inhibitor.

An intriguing finding of this study was the association between increased number of activated basophils in the circulation of PV patients and their complaint of aquagenic pruritus. Pruritus, exacerbated by contact with water during warm bath or shower, is a typical feature of PV reported by up to 65% of patients at diagnosis.[32] It can either ante date diagnosis or appear during the course of disease; it is poorly responsive to phlebotomy or myelosuppressive therapy, while interferon-α,[33,34] or selective serotonin reuptake inhibitors[35] can be successful. Pruritus has been associated with iron deficiency,[36] high leukocyte count,[36] platelet activation,[35] histamine release,[37,38] infiltration of derma by mononuclear cells and eosinophils,[39] and degranulation of dermal mast cells,[39,40] but underlying mechanisms remain substantially obscure. Furthermore, pruritus was more common among patients harboring greater than 50% V617F allele[26,27,41] (and this study); accordingly, we found that circulating activated basophils, measured by their expression of CD63, were significantly increased in this category of patients. Basophils are implicated in immediate hypersensitivity reactions and anaphylaxis, and their granules contain several biogenic amines, including histamine, which might be involved in the pathogenesis of pruritus although no clear correlation of pruritus with plasma histamine levels in PV has been found. However, it is also possible that other *non canonical* mediators such as leukotriene C4 or granzyme B, to name a few,[30,31] might be involved as well as in the pathogenesis of pruritus. Furthermore, basophils can produce and release a vast array of cytokines, such as IL-4, IL-13 and IL-33, which facilitate recruitment and activation of other inflammatory cells (including neutrophils, eosinophils, mast cells); therefore, PV basophils might not necessarily act as effector cells by themselves in causing pruritus.[31,42] Our experimental design does not allow distinguishing among these several possibilities and the mechanistic link between basophils and pruritus requires additional investigation. At this regard, evidence has been provided recently that an increased output of CD34+ cell-derived mast cells in patients with MPN plays a role in pruritogenesis possibly through the release of prostaglandin D2 and increased levels of IL-31.[43]

Overall, results of this study indicated that PV basophils are constitutively activated and hyper-sensitive to IL-3, favoring a direct role of *JAK2V617F* mutation. They also lend support to hypothesizing that activated basophils contribute to pruritus in PV patients and that JAK2 inhibitors might result efficacious to counter this agonizing and treatment-insensitive symptom.

Authorship and Disclosures

LP performed research, analyzed data, and contributed to manuscript writing; CB performed research, analyzed data, and contributed to manuscript writing; PG performed research, analyzed data, and contributed to manuscript writing; MZ performed research, analyzed data; RAR analyzed data and contributed to manuscript writing; NB performed research; AB collected clinical samples and contributed to manuscript writing; AMV designed research, collected clinical samples, analyzed data, and wrote the manuscript.

The authors reported no potential conflicts of interest.

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