

Online supplement

Reagents and antibodies

Hanks buffer and PBS, fMLF, CEP701, protease and phosphatase inhibitors were from Sigma Chemical Co (St. Louis, MO, USA). Recombinant human GM-CSF was from Peprotech (Rocky Hill, NJ, USA). Genistein, AG490, PP1 and PD98059 were from Calbiochem. Endotoxin-free injection-grade water and 0.9% NaCl were from Merck. SDS-PAGE and Western Blot reagents were from Bio-Rad. Anti-phospho-ERK1/2 antibody were from Cell Signaling Technology and anti-ERK1/2 antibodies were from Santa Cruz. Rabbit polyclonal antibodies against p47phox and phospho-serine 345 were previously described ¹.

Ethics Statement

Neutrophils were isolated from venous blood of healthy volunteers and patients with BCR-ABL1-negative chronic MPD managed in the hematology and immunology department of Bichat hospital, Paris, France. The investigations were approved by the local ethics committee and samples were obtained with the volunteers' and patients' written informed consent. All experiments were approved by the INSERM (Institut National de la Santé et de Recherche Médicale) institutional review board and ethics committee. Data collection and analyses were performed anonymously.

Neutrophil preparation

Circulating neutrophils were isolated by Polymorphprep gradient centrifugation ^{1,2}. Briefly, blood was diluted two-fold in sterile PBS and cells were isolated by one-step Polymorphprep

gradient centrifugation at 500 g for 30 minutes at 22°C. The neutrophil band was collected and the cells were washed in PBS and counted.

Patients and diagnostic

We studied 29 patients with BCR-ABL1-negative chronic myeloproliferative disorders managed at the hematology and immunology department of Bichat hospital, Paris, France. Based on WHO diagnostic criteria, 14 patients had ET (7 *JAK2* V617F), 12 PV (10 *JAK2* V617F), 2 PMF (2 *JAK2* V617F) and 1 neutrophilic leukemia (*JAK2* V617F). *JAK2* V617F was detected and quantified by using allele-specific PCR as previously described³. The characteristics of the patients are summarized in Table 1.

Measurement of ROS production

A chemiluminescence method was used for added sensitivity when studying patients' cells: 2.5×10^5 cells were suspended in 0.5 ml of HBSS containing 10 μ M luminol preheated to 37°C in the thermostated chamber of a luminometer (Berthold-Biolumat LB937) and allowed to stabilize. After a baseline reading, cells were stimulated with fMLF (10^{-7} M) and chemiluminescence was recorded^{1,2}.

For superoxide anions measurement, neutrophils (1×10^6) were resuspended in 1 ml of HBSS and treated with AG490 or PD98059 for 30 minutes, then with GM-CSF for 15 minutes at 37°C. Superoxide production was then measured in response to fMLF (10^{-7} M), in terms of superoxide dismutase-inhibitable ferricytochrome c reduction, as described elsewhere^{1,2}.

Flow cytometric analysis of CD11b expression

Whole-blood samples (500 μ l) were incubated at 37°C for 5 minutes with DPBS (resting) or fMLF (10^{-7} M). Then 100 μ l of each sample was incubated with 10 μ l of a PE-conjugated anti-

human CD11b and 10 μ l of FITC-conjugated anti human CD15 monoclonal antibodies (BD Biosciences, San Jose, CA, USA) for 20 minutes at room temperature in the dark. Red cells were lysed with BD FACSTTM lysing solution (BD Biosciences) and white cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Nonspecific antibody binding was determined on cells incubated with the same concentration of an irrelevant antibody of the same isotype. Forward and side scatter were used to identify the neutrophil population and to gate out other cells and debris in a FACS CantoII device (BD Biosciences). The purity of the gated cells was assessed by using a FITC-conjugated anti-CD15 antibody (BD Biosciences). The mean fluorescent intensity (MFI) of CD11b-positive cells was then determined in the neutrophil CD15 positive population. Five thousand events per sample were analysed and all the results were obtained with a constant photomultiplier gain.

P47phox and ERK1/2 phosphorylation in neutrophils

Neutrophils from MPD patients were lysed without stimulation. Neutrophils from healthy donors were treated with kinase inhibitors for 30 minutes, then with GM-CSF (12 ng/ml) at 37°C for 20 minutes. The reaction was stopped by adding ice-cold buffer and by centrifugation at 400 g for 6 minutes at 4°C. The cells were lysed by resuspension in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25 M sucrose, 5 mM EGTA, 5 mM EDTA, 15 μ g/ml leupeptin, 10 μ g/ml pepstatin, 10 μ g/ml aprotinin, 1.5 mM PMSF, 1 mM DFP, 0.5% Triton X-100, 25 mM NaF, 5 mM NaVO₄, 5 mM β -glycerophosphate, 1 mM pNPP, 1 mg/ml DNase I) and sonication on ice (3 x 15 s). The lysate was then centrifuged at 100 000 g for 30 minutes at 4°C in a TL100 ultracentrifuge (Beckman Inc.). Proteins in the cleared supernatant were denatured in Laemmli's sample buffer. The samples were then subjected to SDS-10% PAGE and Western Blot using standard techniques^{4,5}. The intensity of phosphorylated-p47phox, total

p47phox, phosphorylated-ERK and total ERK bands were quantified by densitometry using the Image J analysis program. Phosphorylated intensities were corrected for the corresponding amounts of total protein present on the membrane.

Statistical analysis

All results are expressed as means \pm standard error of the mean (SEM). Significant differences ($p < 0.05$) were identified with Student's *t* test.

References

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