Stat5 is critical for the development and maintenance of myeloproliferative neoplasm initiated by Nf1 deficiency

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Supplemental Information

Materials and Methods

Mice

Genetic backgrounds. *Mx1-Cre* transgenic mice have previously been described\(^1\). The *Stat5a/Stat5b* double knockout mice (*Stat5^ΔN*) were a kind gift from Dr. James Ihle, St. Jude’s Children’s Research Hospital, and have been described previously\(^2^\)-\(^4\). *Nf1^flox^* mice were generously provided by Dr. Luis Parada, University of Texas Southwestern Medical Center\(^5\). The *Nf1^Fcr^* allele has previously been described\(^6\). *Mx1-Cre Nf1^flox/flox^* mice have been described previously \(^7\) and were purchased from The Jackson Laboratory (Bar Harbor, Maine).

Treatment of mice

Recipient C57BL/6J x 129/Sv F1 mice were lethally irradiated in a Mark 1 Model 30 Cs137 irradiator (J.L. Shepherd & Associates, San Fernando, CA) with 900 cGy gamma radiation. Twenty-four hours post irradiation, 5 x10\(^6\) freshly isolated, viable, nucleated donor bone marrow cells were suspended in PBS and injected via the tail vein.

Transplanted animals received three 250 µL intraperitoneal injections in five days of polyinosinc-polycytidylic acid in sterile PBS (1 µg/µL, pIpC, Sigma-Aldrich, St. Louis, MO) one week post-transplant. Ruxolitinib-treated animals (*Nf1^flox/flox^ Mx1-Cre*, with endogenous *Stat5* alleles), received the same pIpC treatment at three weeks of age (after weaning). Adult animals (ages 6-9 months) received twice daily ruxolitinib (10 mg/kg in 5% dimethyl acetamide, 0.05% methylcellulose, Selleckchem, Houston, TX) or vehicle by oral gavage for a period of 6 weeks. Ruxolitinib-treated animals were sacrificed after 6 weeks of treatment or earlier if moribound.
All animals were housed under specific pathogen free conditions. Peripheral blood was obtained by retro-orbital puncture as previously described\textsuperscript{8}. Affected animals were euthanized by CO\textsubscript{2} asphyxiation for analysis. The Institutional Animal Care and Use Committees at the University of Minnesota approved all procedures.

**Human bone marrow and peripheral blood**

The human specimen was obtained with written informed consent in accordance with the policies of the Institutional Review Board of the University of Minnesota. The specimen was de-identified for this study.

**PCR**

PCR was performed on gDNA for the *Nf1 Fcr* and *flox* alleles as described previously\textsuperscript{5,6}.

**Flow and mass cytometry**

Flow and mass cytometry were performed as previously described\textsuperscript{8-10}. Briefly, red blood cells in the spleen and peripheral blood were lysed by suspension in red blood cell lysis buffer (0.15M NH\textsubscript{4}Cl, 1.0M NaHCO\textsubscript{3}, 0.1M Na\textsubscript{2}EDTA). Nucleated cells were suspended in FACS buffer (PBS, 2\% fetal bovine serum, 0.1\% sodium azide). Fc receptors were blocked using anti-CD16/CD32 antibody. Specific antibodies were added, the cells incubated, then washed. Stained cells were resuspended and stored in 1\% formaldehyde in PBS until analysis. Chimerism via Ly5.1/5.2 mismatch was measured for all transplanted animals at the termination of the experiment and necropsy to ensure adequate engraftment. Flow cytometry was performed on a FACS Calibur or LSRII flow cytometer (BD, Franklin Lakes, NJ). Mass cytometry was performed on a CyTOF2
Acquisition and data analysis was performed using CellQuest Pro software (BD) and FlowJo (Tree Star, San Carlos, CA), respectively.

For detection of phospho-specific STAT5 and ERK (pSTAT5 and pERK), intracellular flow cytometry was performed as previously described\textsuperscript{11}. Briefly, murine bone marrow cells were harvested and erythrocytes lysed as described above. Mononuclear cells were incubated then stimulated with murine GM-CSF 10 ng/mL (Peprotech, Rocky Hill, NJ) for the indicated times. The human bone marrow mononuclear cells were isolated using Lymphoprep\textsuperscript{TM} (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacture’s instructions. Cells were fixed with 2% paraformaldehyde at room temperature for 10 minutes. Cells were washed twice with PBS, permeabilized with ice-cold 95% methanol, vortexed, and stored at -20°C overnight or -80°C for longer-term storage.

Samples were washed, incubated at 4°C for 1 hour in FACS buffer (Hanks balanced salt solution containing 4% FBS; Hyclone, Logan, UT), pelleted, then incubated with anti-CD16/CD32 (2.4G2; BD Biosciences, San Jose, CA) on ice for 15 minutes. Samples were washed again, then incubated with the following antibodies in the dark at room temperature for 30 minutes: Alexa 647 conjugated antibody to phospho-STAT5 (Tyr 694; BD Biosciences), phycoerythrin (PE)-conjugated antibody to phospho-ERK (Cell Signaling Technologies), Er167-conjugated antibody to phospho-ERK (Fluidigm), PE-conjugated antibodies to CD3 (17A2), CD4 (GK1.5), B220 (RA3-6B2), CD11b/Mac1 (M1/70), Gr1 (RB6-8C5), and TER-119 (BD Pharmingen), PE-conjugated anti-CD8 (53-6.7) and PE-Cy5– conjugated anti-CD117/c-kit (2B8) (eBioscience, San Diego, CA). Samples were washed once with PBS and analyzed on LSR II flow
cytometer (BD Biosciences). Data were collected using DIVA software (BD Biosciences) and analyzed using FlowJo (Tree Star).

**Complete blood counts**

Murine peripheral blood was analyzed as previously described using a HemaVet Mascot 800 hematology analyzer (CDC Technologies, Inc., Oxford, CT).

**Statistical analysis**

Graphing and statistical analysis was performed with Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

**Supplemental Information References**


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Stat5 insufficiency alleviates MPN in Nf1-deficient mice.

Bone marrow was harvested from donor mice, transplanted into syngeneic recipients, and allowed to engraft (as described in Figure 1). Peripheral blood from transplant recipients was collected every six weeks for the duration of the experiment and with increased frequency in diseased animals. Total white blood cells counts (WBC) and peripheral blood smears (data not shown) were used to monitor the development of myeloproliferative disease in recipient mice. (A) The average hemoglobin concentration of mice from each genotype is shown. Gray-shaded area represents the normal range of hemoglobin concentration. Error bars represent the standard error of the mean. (B) The average platelet count of mice from each genotype is shown. Gray-shaded area represents the normal range of platelet count. Error bars represent the standard error of the mean. (C) Kaplan-Meier survival curve was plotted comparing overall survival of recipients with the indicated genetic background. n ≥7 recipients per group; Logrank followed by Chi Square testing was performed.

Supplemental Figure 2. Ruxolitinib attenuates blood counts and inhibits GM-CSF-mediated induction of STAT5 phosphorylation. Peripheral blood from ruxolitinib and vehicle treated animals was collected every week for the duration of the experiment. The average (A) WBC, (B) hemoglobin concentration, and (C) platelet count of mice from each genotype and treatment group is shown. The gray-shaded area represents the normal range. Error bars represent the standard error of the mean. (D) Bone marrow cells from ruxolitinib- or vehicle-treated mice were treated in vitro with GM-CSF or vehicle for 30
minutes before fixation and permeabilization for intracellular flow cytometry assessment of phospho-STAT5 levels. The induction of phospho-STAT5 levels by GM-CSF, in comparison to basal levels, is shown. Horizontal bars represent the mean; error bars represent the standard error of the mean.
Supplemental Table 1. *Stat5*/*Nf1* deficient mice clinical data. The survival, spleen weight, and autopsy findings of mice with loss of *Stat5* and of *Nf1* alleles.

Supplemental Table 2. *Stat5*/*Nf1* deficient mice white blood cell counts. The WBC of mice with loss of *Stat5* and of *Nf1* alleles, measured throughout the study period.

Supplemental Table 3. *Stat5*/*Nf1* deficient mice hemoglobin concentration. The hemoglobin concentration of mice with loss of *Stat5* and of *Nf1* alleles, measured throughout the study period.

Supplemental Table 4. *Stat5*/*Nf1* deficient mice platelet counts. The platelet counts of mice with loss of *Stat5* and of *Nf1* alleles, measured throughout the study period.

Supplemental Table 5. *Stat5*/*Nf1* deficient mice survival p values. The p values for the survival comparison of mice with different *Stat5Nf1* deficient genotypes.

Supplemental Table 6. Ruxolitinib-treated mice clinical data. The survival and spleen weights of the ruxolitinib-treated mice.

Supplemental Table 7. Ruxolitinib-treated mice white blood counts. The WBC of mice treated with ruxolitinib, measured throughout the study period.
**Supplemental Table 8. Ruxolitinib-treated mice hemoglobin concentration.** The hemoglobin concentration of mice treated with ruxolitinib, measured throughout the study period.

**Supplemental Table 9. Ruxolitinib-treated mice platelet counts.** The platelet counts of mice treated with ruxolitinib, measured throughout the study period.